AD_____

Award Number:
W81XWH-11-1-0754

TITLE: Development of Non-Hormonal Steroids for the Treatment of Duchenne Muscular Dystrophy

PRINCIPAL INVESTIGATOR: Terence Partridge, PhD

CONTRACTING ORGANIZATION: Children's Research Institute
Washington, DC 20010

REPORT DATE: February 2013

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
February 2013	Final	15Sepptember2011–14February2013

4. TITLE AND SUBTITLE
Development of Non-Hormonal Steroids for the Treatment of Duchenne Muscular Dystrophy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-1-0754

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Terence Partridge, PhD
Erica Reeves, PhD
Betty Diamond

E-Mail: Tpartridge@childrensnational.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Children's Research Institute

Washington, DC 20010

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Background: Glucocorticoids are standard of care for Duchenne muscular dystrophy (DMD) patients. The rationale to use glucocorticoids in DMD patients is largely empirical and the molecular mechanisms that help to improve muscle strength in DMD patients are unknown. Despite the success of glucocorticoids in increasing strength, their prescription in DMD remains controversial partly due to significant side effects associated with chronic glucocorticoid use in these patients. It has been proposed that side effects (e.g. metabolic) of glucocorticoids are due to transcriptional activation while beneficial effects (e.g. anti-inflammatory) are due to transrepression indicating that designing selective glucocorticoid receptor (GR) ligands may maintain beneficial effects while reducing metabolic side effects (Coghlan et al., 2003). In collaboration with Validus Biopharma, we have developed glucocorticoid analogues (referred as VBP compounds) that retain the beneficial effects but not the side effects. We have previously shown that these compounds are potent NFκB inhibitors and chronic in vivo administration of these compounds improves skeletal muscle function and histology in the mdx mouse model of DMD, yet reduce side effects commonly associated with existing glucocorticoids. After extensive screening and testing we have identified a lead candidate, VBP15, and preliminary data presented here characterizes the promising ADME and initial safety studies performed to date. Objective/Hypothesis: The objective of this proposal is to characterize the in vitro safety of VBP15 in human hepatocytes, and in vivo non-GLP acute toxicity and GLP 28-day repeated dose toxicity of VBP15 in rat and dog. This data will be used for an investigational new drug (IND) application to the FDA for use of VBP15 in muscular dystrophy. Specific Aims: In this proposal we will perform pre-IND in vitro safety (CYP induction assays in human hepatocytes) and in vivo toxicity studies. The in vivo studies will include non-GLP maximum tolerated dose (MTD)/dose reduction fraction (DRF) and 28-day GLP repeated-dose range-finding in rats and dogs. Study Design: Studies proposed in this application are designed to perform some of the IND enabling safety and toxicology studies with our lead compound VBP15. Relevance: We anticipate that these experiments would provide the basis for future human clinical trials with non-hormonal glucocorticoids not only for DMD but for several human immune and inflammatory diseases. While initially developed for muscular dystrophy, glucocorticoids are in frequent use in the military, and the drugs developed here promise to improve efficacy and reduce side effects of all indications for glucocorticoids.

15. SUBJECT TERMS
Muscular Dystrophy;

16. SECURITY CLASSIFICATION OF: none			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	85	USAMRMC

Table of Contents

	Page
Introduction……..…..	**3**
Body……..	3
Key Research Accomplishments……..	**3**
Reportable Outcomes……	**4**
Conclusion……	**11**
References…….	**12**
Appendices……	**14**

Introduction

Statement of Work (SOW)

We proposed three specific aims, all to be carried out within one year of funding

Aim 1. Perform CYP induction assays in human hepatocytes from 3 donors for CYP3A4 and 1A2 at two doses plus appropriate positive controls.

Aim 2. Perform a pilot non-GLP acute toxicity (single dose) for VBP-15 in mice.

Aim 3. Perform a GLP 28-day repeated-dose range-finding study for VBP15 in mice.

Final Report

Key Research Accomplishments

Our initial rationale of developing the delta 9,11 steroids was to dissociate efficacy from side effects, where retention of NF-κB inhibitory activity was associated with efficacy, and GRE-mediated transcriptional response was associated with side-effects. Ostensibly, the goal was to make a new glucocorticoid without side effects. Previously, we showed data identifying a lead compound (VBP15) for the treatment of DMD. We previously showed data from studies that further characterize the absorption, metabolism, genotoxicity and pharmacokinetics of VBP15. In this funded project, we proposed to further characterize VBP15 on CYP induction and perform IND-enabling toxicology studies.

Aim 1. Perform CYP induction assays in human hepatocytes from 3 donors for CYP3A4 and 1A2 at two doses plus appropriate positive controls.

Cryopreserved human liver microsomes from 3 donors (separate incubations) were obtained commercially from CellzDirect (Invitrogen). Hepatocytes were cultured on a collagen substratum for three days prior to study initiation according to manufacturer's instructions. Hepatocytes cultures were treated daily with fresh media containing 1-, 10-, and 100 μM VBP15, vehicle (negative control) or appropriate positive control (rifampin for CYP3A4 and omeprazole for CYP1A2). 24-hours after the final treatment, CYP3A4 and CYP1A2 activity was determined using the FDA recommended probe substrates testosterone and phenacetin respectively. Assay analysis was conducted via LC/MS/MS. The percentage inductions relative to positive control were calculated. A greater than 40% of positive control in any one of the three donors for a CYP is considered a potential inducer of that CYP. There was no induction of CYP1A2 by VBP15 seen across the three donors. VBP15 is not considered an inducer of CYP1A2. VBP15 moderately induced CYP3A4 across the three donors (24%-42%). This indicates that VBP15 is a potential inducer of CYP3A4.

Aim 2. Perform a pilot non-GLP acute toxicity (single dose) and 7 day repeated dose range finding study for VBP15 in mice.

Study Objective

This study was conducted for ReveraGen BioPharma, Inc., to establish the safety margin, evaluate and characterize the acute toxicity, estimate the maximum tolerated dose (MTD), and evaluate the toxicokinetics of the test article, VBP15, in mice. The study consisted of two phases, Phase A and Phase B. The doses were selected to enable evaluation of toxicity at an exposure comparable or higher than the target exposure for efficacy.

Study Design

During Phase A, four treatment groups of three male and three female Crl:CD1®(ICR) mice were administered the test article at respective dose levels of 50, 125, 250, and 500 mg/kg. The test article was administered to all groups via a single oral gavage dose at a volume of 10 mL/kg.

During Phase B, three treatment groups of 16 male and 16 female Crl:CD1®(ICR) mice were administered the test article at respective dose levels of 10, 30, and 100 mg/kg/day. One additional group of 16 animals/sex served as the control and received the vehicle, Labrafil M 1944 CS. The vehicle or test article was administered to all groups via oral gavage for seven consecutive days at a dose volume of 10 mL/kg. Following seven days of administration, six animals/sex/group were maintained for a seven-day recovery period. Additionally, during

Phase B, three groups of 36 animals/sex/group served as toxicokinetic (TK) animals and received the test article in the same manner and at the same dose levels as the main study groups.

Observations for morbidity, mortality, injury, and the availability of food and water were conducted twice daily for all animals for Phases A and B. Observations for clinical signs were conducted daily for all Phase A animals and all Phase B main study animals. Body weights were measured and recorded daily for all animals for Phases A and B. Food consumption was measured and recorded daily for Phases A and B.

At the termination of Phase A (Day 5), all animals were euthanized and discarded without further evaluations.

During Phase B, blood and urine samples for clinical pathology evaluations were collected from designated main study animals prior to the terminal and recovery necropsies. During Phase B, blood samples for the determination of plasma concentrations of the test article were collected from TK animals at designated time points on Days 1 and 7. After blood collection, the TK animals were euthanized and the carcasses were discarded. At the end of the terminal and recovery periods of Phase B, necropsy examinations were performed, organ weights were recorded, and tissues were microscopically examined.

Reportable Outcomes

During Phase B, no significant effects on:

- clinical observations
- body weight
- hematology
- clinical chemistry
- urinalysis
- macroscopic observations
-

Food consumption: During Phase B, there was a slight test-article decrease in food consumption in males but was not dose dependent (-14 to -20%). In females, there was an increase in consumption at the highest dose (17%). The relationship between treatment and food consumption is unclear due to lack of dose-response in both sexes.

Organ Weight: At the Phase B terminal necropsy, possible test article-related differences in liver, spleen, and thymus weights were observed in treated males and females. All liver weight parameters were higher in males at 50 and 100 mg/kg/day and in females at 100 mg/kg/day, all statistically significant. Spleen weights were statistically significantly lower in males at 100 mg/kg/day and in females in a dose response pattern at all exposure levels. Thymus weights were lower in a dose response pattern for all treated male and female groups (statistically significant at 30 and 100 mg/kg/day for males and females). Organ weights were not statistically different after 7 day recovery period.

Table 1. Organ Weights

Terminal Organ Weights		0 mpk/day		10 mpk/day		30 mpk/day		100 mpk/day	
Organ	**Gender**	**Mean (g)**	**SD**	**Mean (g)**	**SD**	**Mean (g)**	**SD**	**Mean (g)**	**SD**
Liver	M	1.371	0.251	1.413 3.06%	0.251	1.647 20.13%	0.244	1.997[b] 45.66%	0.337
Spleen	M	0.073	0.016	0.070 -4.11%	0.017	0.069 -5.48%	0.011	0.054[b] -26.03%	0.009
Thymus	M	0.050	0.020	0.044 -12.00%	0.015	0.029[b] -42.00%	0.008	0.023 -54.00%	0.008
- Increase in leukocytes at 30 mpk/day - Increase in neutrophils at 30 and 100 mpk/day - No change in lymphocytes									
Terminal Organ Weights		**0 mpk/day**		**10 mpk/day**		**30 mpk/day**		**100 mpk/day**	
Organ	**Gender**	**Mean (g)**	**SD**	**Mean (g)**	**SD**	**Mean (g)**	**SD**	**Mean (g)**	**SD**
Liver	F	1.142	0.235	1.117 -2.19%	0.213	1.168 2.28%	0.169	1.455[b] 27.41%	0.191
Spleen	F	0.105	0.037	0.077[a] -26.67%	0.016	0.070[b] -33.33%	0.014	0.063[b] -40.00%	
Thymus	F	0.069	0.018	0.059 -14.49%	0.019	0.032[b] -53.62%	0.012	0.022[b] -68.12%	0.005
- No change in leukocytes, neutrophils or lymphocytes									

Toxicokinetic Summary

Evidence of systemic exposure to VBP15 was observed in VBP15-treated mice following both single and repeat daily PO administration of VBP15. Over the dose range evaluated, mean T_{max} values were dose-independent and observed at the first post-dose collection time point of 1.00 hour for females and males following PO administration of VBP15 on Study Days 1 and 7. VBP15 exposure increased with increasing dose on Study Days 1 and 7. The observed increases in AUC_{last} over the full dose range (10 – 100 mg/kg/day) were greater than proportional to dose (i.e., ≥25% of the fold increase in dose) on Study Days 1 and 7. Despite occasional differences, review of VBP15 plasma concentrations and resulting TK parameters revealed no clear or consistent evidence of a gender difference. Repeat, PO dosing of VBP15 over the study duration was associated with decreases in mean plasma VBP15 AUC_{last} values (ranging from 1.92 to 2.81-fold reduced) for female and male mice on Study Day 7 compared to Study Day 1 values, with the exception of female mice at the 10 mg/kg/day dose level (1.70-fold increase). The actual cause for the reduction on the Day 7 exposure is unknown, while some possible explanations include metabolic induction or oral absorption reduction. Additional investigations may be needed in order to fully understand the underlying mechanism(s) for such phenomenon.

Histopath Analysis

- Spleen: No microscopic correlates identified.

- Liver: The liver had minimal to mild panlobular hepatocyte hypertrophy in males and females at 100 mg/kg/day and minimal individual hepatocyte necrosis in two (2 of 10) males at 100 mg/kg/day. Due to the presence of concurrent individual hepatocyte necrosis, this dose was considered to be adverse in males. The liver also had minimal to moderate centrilobular vacuolation or minimal periportal vacuolation in males at 30 and 100 mg/kg/day and females at 100 mg/kg/day. Centrilobular vacuolation

predominantly involved centrilobular to midzonal hepatocytes and was characterized by clearing of the perinuclear cytoplasm with irregularly-shaped vacuoles that had poorly defined margins. Centrilobular vacuoles demonstrated some positive staining for Periodic acid-Schiff (PAS), negative staining with PAS and diastase, and some positive staining with Oil red O which suggested that glycogen and lipid likely contributed to the vacuolated appearance; however, it was not clear whether glycogen and lipid were solely responsible for the centrilobular vacuolation. Periportal vacuolation predominantly involved periportal to midzonal hepatocytes and was characterized by one or more, small, round, clear, distinct vacuoles within the cytoplasm. Periportal vacuoles stained positively for Oil red O and negatively for PAS with and without diastase indicating that periportal vacuolation was due to the presence of lipid. Minimal focal necrosis was present at a similar incidence in control and treated animals at 100 mg/kg/day; therefore this finding was not considered to be test article related.

- Thymus: The thymus had non-adverse minimal to mild generalized lymphoid depletion in males and females at 100 mg/kg/day. Minimal generalized lymphoid depletion was present in males at all dose levels including controls and in one female (1 of 10) at 30 mg/kg/day. At 30 mg/kg/day, thymic weights were statistically lower in males and females and the incidence of generalized lymphoid depletion was slightly increased in males and females; however, it was unclear whether this slight increase in incidence was test article-related at this dose.

Aim 3. Perform a non-GLP 28-day repeated-dose range-finding study for VBP15 in mice.

Mouse 28-day Repeat Dose Study. 1998-003. MPI

Study Objective

The objective of this study was to evaluate the potential subchronic toxicity of the VBP15 in mice following 4 weeks of oral administration and to evaluate reversibility, progression, or delayed appearance of any changes following a 2-week post-dose observation period.

Table 2: Study Design

Species/Strain	Method of Administration (Vehicle/ Formulation)	Duration of Dosing	Dose Level (mg/kg/day)	Number/ Sex/Group	NOAEL (mpk/day)
Mouse/Crl:CD1®(ICR)	Oral Gavage/ Labrafil M 1944 CS (Gattefosse Labrafil)	Once daily for 28 days followed by a 14-day recovery period	0, 10, 30, 100	Main Study: 10M, 10F Recovery: 5M, 5F Toxicokinetics: 9M, 9F (0 mg/kg/day) 39 M, 39F (10, 30, 100 mg/kg/day)	At least 100

Table 3. Toxicokinetic Parameters (combines sexes).

Dose Level (mg/kg/day)	0	10[dose]	30[dose]	100[dose]
Day 1				
C_{max} (ng/mL)	NA	624	3590	12100
T_{max} (hr)	NA	1.00	1.00	2.00
AUC_{all} (hr•ng/mL)	NA	2000	15800	105000
Day 28				
C_{max} (ng/mL)	NA	548	2930	5840
T_{max} (hr)	NA	2.00	1.00	1.00
AUC_{all} (hr•ng/mL)	NA	1560	7200	34400

Noteworthy Findings
All Treated Groups

- Reduced body weight gain, less pronounced in males than females. Resolved during recovery except for 100 mpk males.
- Increased food consumption during recovery.
- Reduced lymphocytes in lymphoid tissues (mandibular and mesenteric lymph nodes, and thymus). Resolved during recovery.
- Increased thymus weights at recovery.
- Reduced adrenal gland weights, variable between groups and without a dose response relationship.
- Degenerative and atrophic changes in adrenal cortex. Resolved during recovery.

Body and Organ Weights

Terminal

Group mean terminal body weights for females given 100 mpk were statistically significantly decreased compared to animals of the control group.

Differences in group mean organ weights between VBP15 (males and/or females) and the respective control groups were present in the adrenal glands, kidneys, liver, ovaries, pituitary, spleen, and/or thymus. Microscopic findings correlating to organ weight differences were present in the adrenal glands, livers, spleen, and thymus of males and females. All other organ weight differences were considered to be potentially VBP15-related but not adverse as there were no adverse microscopic findings indicative of injury.

Group mean adrenal gland weights were variable between groups and generally decreased but without a dose response relationship. Absolute group mean adrenal weight for females given 100 mpk were statistically significantly decreased and females given 10 or 30 mpk had decreased group mean adrenal weights although the decreases were not dose dependent. Males given 100 mpk had comparable group mean adrenal weights comparable to the control group whereas group mean adrenal weights for males given 10 or 30 mpk were decreased. Adrenal gland organ weight changes were considered to be VBP15-related and correlated microscopically with degenerative and atrophic changes in the adrenal cortex.

Group mean liver weights (absolute, relative to brain weight, and relative to body weight) were statistically significantly increased in males and females given 100 mpk. Males given 30 mpk also had increased liver weights but only liver weight relative to body weight was statistically significantly increased. Microscopic findings in the liver potentially correlating to increased weights included centrilobular hepatocellular hypertrophy, increased hepatocellular vacuolation, and hepatocellular necrosis (single cell and/or subcapsular).

Group mean spleen weights (absolute, relative to brain weight, and relative to body weight) were statistically significantly decreased in males and females given 30 or 100 mpk and group mean spleen weights were decreased (not statistically significantly decreased) for males and females given 10 mpk. Generalized atrophy of the spleen correlated with the decreased weights.

Group mean thymus weights (absolute, relative to brain weight, and relative to body weight) were statistically significantly decreased in males and females given 100 mpk and in females given 30 mkp. Group mean thymus weights were decreased (not statistically significantly decreased) for males given 10 or 30 mpk. Decreased thymus weights correlated with decreased lymphocytes.

Group mean absolute kidney weights were minimally decreased in females given 30 or 100 mpk. There were no microscopic observations that correlated to the decreased kidney weights in females and at least a portion of the decreased absolute weight was likely associated with decreased body weight.

Group mean pituitary weights (absolute, relative to brain weight, and relative to body weight) were decreased in males given 30 or 100 mpk. There were no microscopic observations that correlated to the decreased pituitary weights.

Dose-related decreased group mean ovary weights (absolute, relative to brain weight, and relative to body weight) were present in females given the VBP15. The significance of this organ weight change is uncertain as there were no microscopic findings that correlated to the decreased ovarian weights and there were no apparent effects on the estrous cycle based on staging of the uterus and vagina in females given 100 mpk.

Table 4. VBP15-related organ weight changes- Terminal. Percent change to control.

Dose level: mg/kg/day	10		30		100	
Sex	M	F	M	F	M	F
Number Examined	10	10	10	10	10	10
Body weight (g)	↓ 1.25	↓ 4.96	↓ 5.61	↓ 6.11	↓ 5.30	↓ 9.54[a]
Adrenal glands (g)	↓ 11.11	↓ 18.18	↓ 55.56	↓ 9.09	0.00	↓ 36.36[a]
Adrenal gland/BWt (%)	↓ 7.02	↓ 14.58	↓ 49.82	↓ 8.56	↑0.35	↓ 36.34
Kidneys (g)	-	-	-	↓ 10.33	-	↓ 11.43[a]
Kidneys/BWt (%)	-	-	-	↓ 4.66	-	↓ 2.17
Liver (g)	-	-	↑ 11.03	-	↑ 45.94[b]	↑ 22.87[a]
Liver/BWt (%)	-	-	↑ 18.10[b]	-	↑ 55.29[b]	↑ 36.39[b]
Ovaries (g)	-	↓ 15.79	-	↓ 21.05[a]	-	↓ 31.58[b]
Ovaries/BWt (%)	-	↓ 11.96	-	↓ 19.22	-	↓ 25.54[a]
Pituitary (g)	-	-	↓ 12.09	-	↓ 16.13[a]	-
Pituitary/BWt (%)	-	-	↓ 7.22	-	↓ 12.37	-
Spleen (g)	↓ 14.43	↓ 12.84	↓ 41.24[b]	↓ 29.36[b]	↓ 48.45[b]	↓ 45.87[b]
Spleen/BWt (%)	↓ 13.78	↓ 8.28	↓ 37.28[b]	↓ 23.94[b]	↓ 45.14[b]	↓ 40.84[b]
Thymus (g)	↓ 28.26	-	↓ 32.61	↓ 30.43[b]	↓ 56.52[b]	↓ 67.39[b]
Thymus/BWt (%)	↓ 28.53	-	↓ 28.11	↓ 24.91[a]	↓ 55.38[b]	↓ 63.45[b]

[a]Significantly different from control; ($p<0.05$)
[b]Significantly different from control; ($p<0.01$)
BWt - Body Weight

↑ - Increased
↓ - Decreased
M – Male
F – Female

Recovery

There were no statistically significant differences in group mean terminal body weights.

Group mean thymus weights were increased for all VBP15-treated groups after the recovery period. Group mean thymus weights (absolute, relative to body weights, and relative to brain weight) were statistically significantly increased for females of all VBP15-treated groups and for males given 30 mpk or 100 mpk. The increased thymus weights in males given 30 mpk or 100 mpk correlated with generalized increased lymphocytes. The increased thymus weights likely represent a compensatory increase in lymphocytes in response to VBP15-related decreased lymphocytes present at the terminal necropsy.

Females given 100 mpk had decreased (statistically significantly decreased for absolute and relative to brain weight) group mean right submandibular/sublingual salivary gland weights. The potential relationship to previous exposure to the VBP15 is unknown but the decreased weights were not considered adverse as there were no microscopic observations associated with the decreased weights.

Table 5. VBP15-related Organ Weight Changes- Recovery. Percent change relative to control

Dose level: mg/kg/day	10		30		100	
Sex	M	F	M	F	M	F
Number Examined	10	10	10	10	10	10
Body weight (g)	↓ 2.69	↓ 3.31	↓ 0.30	↓ 2.21	↓ 5.39	↓ 4.04
Sal.gl, man./sub., rt (g)	-	-	-	-	-	↓ 23.08[a]
Sal.gl, man./sub., rt /BWt (%)	-	-	-	-	-	↓ 19.92
Thymus (g)	↑ 50.00[a]	↑17.31	↑83.33[b]	↑48.08	↑72.22[b]	↑53.85[a]
Thymus/BWt (%)	↑ 53.09[a]	↑21.21	↑81.11[b]	↑49.95	↑81.66[b]	↑58.73[a]

[a]Significantly different from control; ($p<0.05$)
[b]Significantly different from control; ($p<0.01$)
BWt - Body Weight

↑ - Increased
↓ - Decreased
M – Male
F - Female

Clinical Chemistry

At termination, both sexes receiving 100 mpk had mild increases in aspartate aminotransferase (AST) (up to 2.0-fold) and alanine aminotransferase (ALT) (up to 3.5-fold), relative to controls, typical of hepatocellular effects. These changes correlated with microscopic changes in the liver (centrilobular hypertrophy, hepatocellular vacuolation and single-cell and subcapsular necrosis) were considered VBP15-related but not adverse based on their magnitude.

Hematology

At termination, both sexes receiving ≥ 10 mpk had mild reductions in lymphocytes (up to 49%), relative to controls. These changes were mostly dose-dependent and correlated with microscopic changes in the mandibular and mesenteric lymph nodes and thymus (decreased lymphocytes). Decreases in lymphocytes were considered VBP15-related but not adverse based on their magnitude.

Toxicokinetics

Dose formulation sample analysis revealed that in general the dose formulations were within the expected variability of their targeted concentrations, had minimal to no stratification and were stable for the 20 day test period under the conditions of this study. It was noted that the top and middle strata samples for the 10 mg/mL (100 mpk dose level) were outside of the expected 25% variability and might indicate some animals in this dose group during Week 1 may have received slightly higher than targeted dose levels. However, this is not felt to have a meaningful impact on the TK analysis and interpretation of this study.

All plasma concentrations of VBP15 in the vehicle control group samples were BLQ. Evidence of systemic plasma exposure to VBP15 was observed in all VBP15-treated groups following both single and repeat PO administration. Despite occasional differences, no clear or persistent evidence of a gender difference was observed in VBP15 plasma concentrations or resulting TK parameter values. VBP15 absorption (as assessed by T_{max}) following PO administration of VBP15 at 10, 30, and 100 mpk appeared to be dose-independent on Day 1 and Day 28 **(Figure 1, Figure 2**).

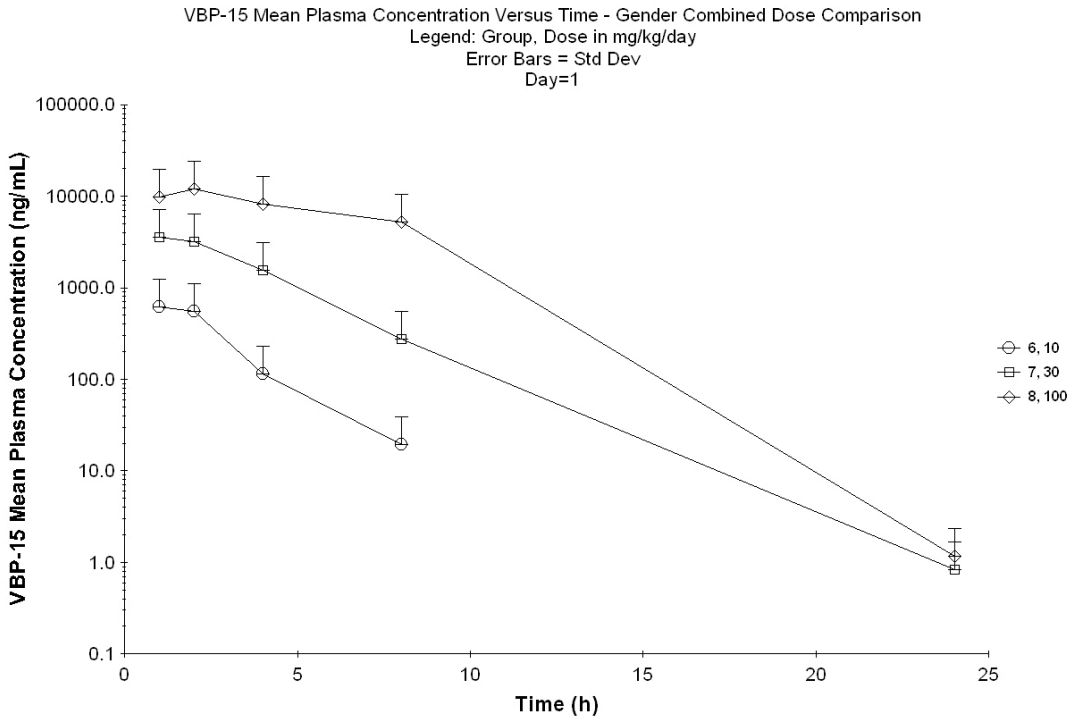


Figure 1. VBP15 Mean plasma concentration versus time- Day 28. Combined gender dose comparison.

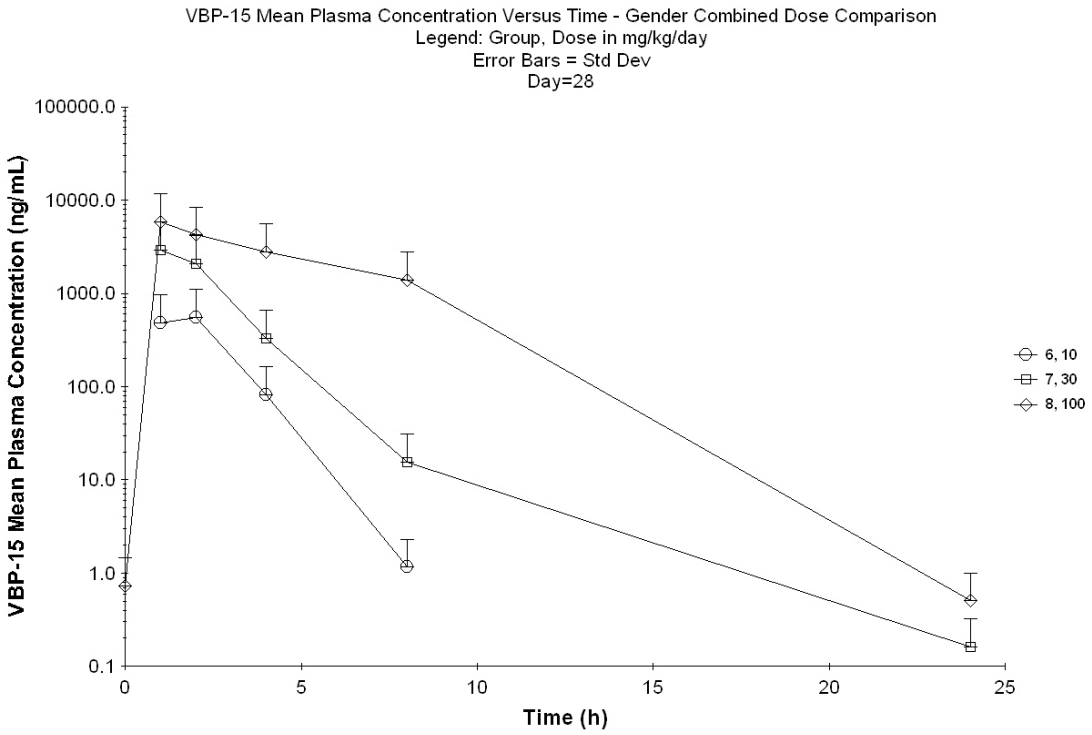


Figure 2. VBP15 mean plasma concentration versus time- Day 1. Combined gender dose comparison.

Exposure as assessed by C_{max} and AUC_{all} increased with increasing dose. Over the full dose range evaluated (10 to 100 mpk) the observed increases in C_{max} were greater than proportional to dose on Day 1 and reasonably proportional to dose on Day 28. The observed increases in AUC_{all} were greater than proportional to dose on Days 1 and 28. Repeat, daily PO administration of VBP15 over a 28-day duration was associated with decreases in mean VBP15 AUC_{all} values, consistent possible enzyme induction under the conditions of this study. These data suggests CYP induction thus inhibiting its own metabolism over in time.

Table 6. Group Mean VBP15 Pharmacokinetic Summary Data; Sorted by Compound, Route, Day, Group, Dose, and Gender

Compound	Route	Day	Group	Dose (mg/kg)	Gender	C_{max} (ng/mL)	SE C_{max} (ng/mL)	T_{max} (h)	AUC_{all} (h*ng/mL)	SE AUC_{all} (h*ng/mL)	AUC_{INFobs} (h*ng/mL)	$HL_{\lambda z}$ (h)
VBP15	PO	1	6	10	Female	379	331	2.00	1110	556	1090	0.796
					Male	966	78.8	1.00	2890	397	2670	1.43
					F+M	624	176	1.00	2000	373	1880	1.33
			7	30	Female	3240	619	2.00	10800	2180	10700	0.725
					Male	4480	727	1.00	20800	1750	20800	1.93
					F+M	3590	787	1.00	15800	1900	15800	1.85
			8	100	Female	12800	1500	2.00	115000	3460	115000	1.50
					Male	11400	917	2.00	95200	18900	95200	1.56
					F+M	12100	845	2.00	105000	8980	105000	1.49
		28	6	10	Female	577	299	1.00	1380	313	1370	0.628
					Male	607	102	2.00	1750	349	1740	0.712
					F+M	548	59.0	2.00	1560	232	1560	0.672
			7	30	Female	3330	964	1.00	7870	1320	7870	2.14
					Male	2530	218	1.00	6530	426	6460	0.772
					F+M	2930	477	1.00	7200	651	7200	1.65
			8	100	Female	5220	1200	1.00	39200	6890	NR	NR
					Male	6450	919	1.00	29500	6100	29500	1.84
					F+M	5840	728	1.00	34400	4400	34400	1.54

Discussion

The data from this study show that VBP15 produces many of the same effects in normal (wild-type) mice that are known to be associated with exogenous administration of synthetic glucocorticoids, but it requires much higher doses of VBP15 to cause these effects. These effects include reduction in body weight; effects on organ weights in the liver, thymus, spleen and adrenal glands; microscopic lesions on the liver, spleen and adrenal glands; and changes in circulating lymphocytes, AST and ALT levels.

Females at the highest dose tested significantly decreased body weight on Day 28 which reversed itself by the end of the recovery phase (Day 42). It is well known that chronic administration of glucocorticoids in humans is associated with increased body weight due to increased fat disposition and appetite stimulation. However, chronic administration of glucocorticoids in mice is associated with decreased body weight (Sali et al., 2012; Mizunoya et al., 2011). In these reports, the decrease in body weight occurs at much lower doses compared to VBP15 (i.e. 5 mpk of prednisone compared to 100 mpk VBP15) which is consistent with the data obtained in the mdx mouse model presented above.

In many species, including humans, exogenous administration of glucocorticoids is known to exert a negative feedback effect on the hypothalamic-pituitary-thyroid axis resulting in adrenal suppression (Kaptein et al., 1992; Davies & Franklin, 1984). Rodents are not a good species to study the effects of exogenous glucocorticoids on adrenal suppression however, the effect on adrenal weight could be used as an indicator for potential suppression. In this study, VBP15 generally decreased adrenal weights but without a dose response relationship. Adrenal gland organ weight changes were considered to be VBP15-related and correlated microscopically with degenerative and atrophic changes in the adrenal cortex.

Decreases in lymphocyte counts were observed with VBP15. These changes were mostly dose-dependent and correlated with microscopic changes in the lymph nodes and thymus. These changes in lymphocyte counts were considered VBP15-related but not adverse based on their magnitude. Glucocorticoids are known to decrease peripheral blood lymphocyte count. This is an anticipated response based on the pharmacology of both VBP15 and prednisone as both compounds are immune modulators via NF-κB inhibition.

VBP15 mildly increased serum ALT and AST levels. The microscopic abnormalities observed in the liver, including centrilobular hepatocellular hypertrophy, increased hepatocellular vacuolation, and hepatocellular necrosis are consistent with the hepatic lesions reported by other investigators in beagles treated with glucocorticoids (Kuhlenschmidt et al., 1991; Nagashima et al., 1992). It is likely that the hepatocyte hypertrophy observed in due at least in part to hepatic drug metabolizing enzymes. It has been reported that increases in hepatic microsomal activity after administration of investigative drugs was associated with increased liver weight and hepatocellular hypertrophy (Amacher et al., 2001). However, these authors did not find correlation between hepatic enzyme induction and increased levels of liver enzymes, such as ALT and AST indicative of hepatotoxicity. Therefore is appears that the increased levels of serum liver enzymes in response to drug can occur independently of microsomal enzyme induction and liver injury.

Conclusion

Based on the absence of adverse effects following 4 weeks of oral administration of VBP15 to mice at 10, 30, and 100 mpk/day, the No-Observable-Adverse-Effect Level (NOAEL) is considered to be at least 100 mpk/day. Note that pre-clinical studies in the *mdx* showed optimal doses at 30 mpk/day.

References

1: Heier CR, Damsker JM, Yu Q, Dillingham BC, Huynh T, Van der Meulen JH, Sali A, Miller BK, Phadke A, Scheffer L, Quinn J, Tatem K, Jordan S, Dadgar S, Rodriguez OC, Albanese C, Calhoun M, Gordish-Dressman H, Jaiswal JK, Connor EM, McCall JM, Hoffman EP, Reeves EK, Nagaraju K. VBP15, a novel anti-inflammatory and membrane-stabilizer, improves muscular dystrophy without side effects. EMBO Mol
Med. 2013 Oct;5(10):1569-85. Epub 2013 Sep 9. PubMed PMID: 24014378; PubMed Central PMCID: PMC3799580.

2: Damsker JM, Dillingham BC, Rose MC, Balsley MA, Heier CR, Watson AM, Stemmy EJ, Jurjus RA, Huynh T, Tatem K, Uaesoontrachoon K, Berry DM, Benton AS, Freishtat RJ, Hoffman EP, McCall JM, Gordish-Dressman H, Constant SL, Reeves EK, Nagaraju K. VBP15, a glucocorticoid analogue, is effective at reducing allergic lung inflammation in mice. PLoS One. 2013 May 7;8(5):e63871. Print 2013. PubMed PMID: 23667681; PubMed Central
PMCID: PMC3646769.

3: Reeves EK, Hoffman EP, Nagaraju K, Damsker JM, McCall JM. VBP15: preclinicalcharacterization of a novel anti-inflammatory delta 9,11 steroid. Bioorg Med Chem. 2013 Apr 15;21(8):2241-9. Epub 2013 Feb 18.
PubMed PMID: 23498916.

4 Hoffman EP, Reeves E, Damsker J, Nagaraju K, McCall JM, Connor EM, Bushby K. Novel approaches to corticosteroid treatment in Duchenne muscular dystrophy. Phys Med Rehabil Clin N Am. 2012 Nov;23(4):821-8. j.pmr.2012.08.003. Review. PubMed PMID: 23137739; PubMed Central PMCID: PMC3606917.

5 Baudy AR, Reeves EK, Damsker JM, Heier C, Garvin LM, Dillingham BC, McCall J, Rayavarapu S, Wang Z, Vandermeulen JH, Sali A, Jahnke V, Duguez S, DuBois D, Rose MC, Nagaraju K, Hoffman EP. Δ-9,11 modification of glucocorticoids dissociates nuclear factor-κB inhibitory efficacy from glucocorticoid response element-associated side effects. J Pharmacol Exp Ther. 2012 Oct;343(1):225-32.
Epub 2012 Jun 28. PubMed PMID: 22743576; PubMed Central PMCID: PMC3464029.

1521-0103/12/3431-225–232$25.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS Vol. 343, No. 1

194340/3792647
JPET 343:225–232, 2012

Δ-9,11 Modification of Glucocorticoids Dissociates Nuclear Factor-κB Inhibitory Efficacy from Glucocorticoid Response Element-Associated Side Effects[S]

Andreas R. Baudy, Erica K. M. Reeves, Jesse M. Damsker, Christopher Heier, Lindsay M. Garvin, Blythe C. Dillingham, John McCall, Sree Rayavarapu, Zuyi Wang, Jack H. Vandermeulen, Arpana Sali, Vanessa Jahnke, Stephanie Duguez, Debra DuBois, Mary C. Rose, Kanneboyina Nagaraju, and Eric P. Hoffman

Department of Integrative Systems Biology, George Washington University School of Medicine and Health Sciences, Washington, District of Columbia (A.R.B., L.M.G., S.R., Z.W., M.C.R., K.N., E.P.H.); Center for Genetic Medicine Research, Children's National Medical Center, Washington, District of Columbia (A.R.B., C.H., L.M.G., S.R., Z.W., J.H.V., A.S., V.J., S.D., M.C.R., K.N., E.P.H.); ReveraGen BioPharma, Rockville, Maryland (E.K.M.R., J.M.D., B.C.D.); PharMac LLC, Boca Grande, Florida (J.M.); and Department of Pharmaceutical Sciences, State University of New York at Buffalo, Buffalo, New York (D.D.)

Received March 14, 2012; accepted June 25, 2012

ABSTRACT

Glucocorticoids are standard of care for many inflammatory conditions, but chronic use is associated with a broad array of side effects. This has led to a search for dissociative glucocorticoids—drugs able to retain or improve efficacy associated with transrepression [nuclear factor-κB (NF-κB) inhibition] but with the loss of side effects associated with transactivation (receptor-mediated transcriptional activation through glucocorticoid response element gene promoter elements). We investigated a glucocorticoid derivative with a Δ-9,11 modification as a dissociative steroid. The Δ-9,11 analog showed potent inhibition of tumor necrosis factor-α-induced NF-κB signaling in cell reporter assays, and this transrepression activity was blocked by 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-one (RU-486), showing the requirement for the glucocorticoid receptor (GR). The Δ-9,11 analog induced the nuclear translocation of GR but showed the loss of transactivation as assayed by GR-luciferase constructs as well as mRNA profiles of treated cells. The Δ-9,11 analog was tested for efficacy and side effects in two mouse models of muscular dystrophy: *mdx* (dystrophin deficiency), and *SJL* (dysferlin deficiency). Daily oral delivery of the Δ-9,11 analog showed a reduction of muscle inflammation and improvements in multiple muscle function assays yet no reductions in body weight or spleen size, suggesting the loss of key side effects. Our data demonstrate that a Δ-9,11 analog dissociates the GR-mediated transcriptional activities from anti-inflammatory activities. Accordingly, Δ-9,11 analogs may hold promise as a source of safer therapeutic agents for chronic inflammatory disorders.

Introduction

Glucocorticoids have been studied extensively for the past 60 years and are among the most prescribed drugs (Hillier, 2007). They exhibit potent anti-inflammatory properties and are standard of care for many chronic and acute inflammatory conditions, including lupus, myositis, asthma, rheumatoid arthritis, and muscular dystrophy (Baschant and Tuckermann, 2010). However, the side-effect profiles of pharmacological glucocorticoids are significant, including muscle atrophy, adrenal deficiency, osteoporosis, spleen atrophy, short stature, and mood and sleep disturbances, among others (Chrousos et al., 1993; DeBosscher, 2010). This has led to a search for dissociative steroids—drugs able to retain activities responsible for molecular and clinical efficacy with the loss of subactivities responsible for side effects (Newton and Holden, 2007).

The mechanism of action of glucocorticoids is through transrepression and transactivation properties. Transrepression

This work was supported in part by grants from the National Institutes of Health *Eunice Kennedy Shriver* National Institute of Child Health and Human Development [Grant U54-HD053177] (Wellstone Muscular Dystrophy Research Center); the National Institutes of Health *Eunice Kennedy Shriver* National Institute of Child Health and Human Development [Grant 5R24-HD050846] (National Center for Medical Rehabilitation Research); the National Institutes of Health *Eunice Kennedy Shriver* National Institute of Child Health and Human Development [Grant 1P30-HD40677] (Intellectual and Developmental Disabilities Research Center); the National Institutes of Health National Institute of Arthritis and Musculoskeletal and Skin Diseases [Grant R01-AR050478]; the National Institutes of Health National Heart, Lung, and Blood Institute [Grant R01-HL033152]; the Foundation to Eradicate Duchenne; the Muscular Dystrophy Association Venture Philanthropy; the Myositis Association; and the U.S. Department of Defense [Grants W81XWH-05-1-0616, W81XWH-04-01-0081].

A.R.B., E.K.M.R., and J.M.D. contributed equally to this work.

Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.

http://dx.doi.org/10.1124/jpet.112.194340.

[S] The online version of this article (available at http://jpet.aspetjournals.org) contains supplemental material.

involves ligand/receptor interactions with other cellular signaling proteins, such as the inhibition of nuclear factor-κB (NF-κB) complexes (Rhen and Cidlowski, 2005; Newton and Holden, 2007). Transrepression has been associated with anti-inflammatory activity and clinical efficacy. Transactivation (also termed *cis*-regulation) is mediated by ligand/glucocorticoid receptor (GR) translocation from the cytoplasm to the nucleus, with ligand/receptor dimers binding directly to glucocorticoid response elements (GREs) in the promoters of target genes (Dostert and Heinzel, 2004). Transactivation has been associated with deleterious side effects (Newton and Holden, 2007).

The balance of efficacy and side effects is well illustrated in the effects of glucocorticoids on muscle tissue. Chronic administration of glucocorticoids leads to relatively rapid muscle loss via the Forkhead box O (FOXO)/atrogin atrophy signaling pathway, and can cause glucocorticoid myopathy (critical care myopathy) (Di Giovanni et al., 2004; Puthucheary et al., 2010). In contrast, chronic glucocorticoids are used in Duchenne muscular dystrophy to increase muscle strength and prolong ambulation, possibly through their anti-inflammatory effect (Bach et al., 2010; Hussein et al., 2010; Escolar et al., 2011). The beneficial properties of steroids are offset partly by side effects, including atrogin-mediated muscle wasting bone fragility, obesity, and short stature (Schara et al., 2001).

Lazaroid Δ-9,11 analogs were developed originally as non-glucocorticoid steroids with effects on cell membranes and tested clinically for neuroprotection by the inhibition of lipid peroxidation (Taylor et al., 1996; Bracken et al., 1997; Kavanagh and Kam, 2001). More recent studies of lazaroids in muscle found the inhibition of calcium release in C_2C_{12} muscle cells (Passaquin et al., 1998) and the attenuation of ischemia/reperfusion injury (Campo et al., 1997). Lazaroids have been found to protect against acute inflammation (endotoxin-induced shock) through the inhibition of inducible nitric-oxide synthase (Altavilla et al., 1999), tumor necrosis factor-α (TNF-α) (Altavilla et al., 1998), and NF-κB (Fukuma et al., 1999). A Δ-9,11 analog without the large polar 21-amino group has been developed as an anti-neovascularization agent in macular degeneration (anecortave) (Clark, 2007) and more recently investigated for a similar effect in retinoblastoma (Bajenaru et al., 2010). In this article, we investigated a Δ-9,11 analog (anecortave; prodrug), as well as its active metabolite [anecortave desacetate (VBP1)], as a dissociative steroid.

Materials and Methods

Mice. C57BL/6, SJL/J (dysferlin deficient), and C57BL/10ScSn-Dmd^{mdx}/J (*mdx*) (dystrophin deficient) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). GR(dim/dim) mice (Reichardt et al., 1998) were obtained from Deutsches Krebsforschungszentrum (German Cancer Research Center, Heidelberg, Germany). All of the animal experiments were conducted in accordance with our Institutional Animal Care and Use Committee guidelines under approved protocols.

Synthesis of Δ-9,11 Analogs. Analogs were synthesized by Bridge Organics (Kalamazoo, MI) (Supplemental Fig. 1). Anecortave is the prodrug for VBP1.

NF-κB Inhibition Assay. The in vitro drug screening assay for NF-κB inhibition in C_2C_{12} cells was done as described previously (Baudy et al., 2009). To measure the ablation of NF-κB effects by the addition of a GR antagonist, reporter cells were pretreated for 1 h with the drug at a constant concentration and 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-one (RU-486) at increasing concentrations (1 nM to 10 μM). After the pretreatment, cells were stimulated with TNF-α (10 ng/ml) and assayed for luciferase activity 3 h later.

For studies using GR(dim/dim) mice, splenocytes were isolated from GR(dim/dim) and C57BL/6 mice. Splenocytes were treated with prednisolone (10 μm), anecortave (10 μM), or vehicle [dimethyl sulfoxide (DMSO)] for 24 h. After the treatment, cells were stimulated with TNF-α (10 ng/ml) for an additional 24 h. A murine NF-κB-regulated cDNA plate array (Signosis, Inc., Sunnyvale, CA) was used to monitor gene expression.

Nuclear Translocation Assays. Translocation assays were performed by DiscoveRx (Fremont, CA) using GR, mineralocorticoid receptor (MR), and androgen receptor (AR) Nuclear Translocation PathHunter cells (DiscoveRx).

Immunofluorescence. A549 cells were incubated with the drug for 24 h in serum-free media, fixed using formaldehyde, and then processed with primary antibody (rabbit anti-GR; 1:50; GGR H-300; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Alexa Fluor 568 goat anti-rabbit IgG1:100 (Invitrogen, Carlsbad, CA), and 4,6-diamidino-2-phenylindole were used for visualization on an LSM 510 confocal microscope (Carl Zeiss Inc., Thornwood, NY).

GRE Transcription Assay. HEK293 cells stably transfected with a luciferase reporter construct regulated under a GRE (Panomics, Fremont, CA) were grown according to the manufacturer's instructions. Cells were treated with drugs for 6 h at 37°C, and luciferase activity was measured (Promega, Madison, WI) using a Centro LB 960 luminometer (Berthold Technologies, Bad Wildbad, Germany).

Myotube Microarrays. H-2K myoblasts were obtained as described previously (Morgan et al., 1994; Harris et al., 1999). Conditionally, immortalized wild-type and *mdx* H-2K myoblast cell lines underwent differentiation to myotubes for 5 days and were exposed to prednisolone, anecortave, or DMSO vehicle for 4 h. RNA was isolated and analyzed on Affymetrix 430a 2.0 microarrays. Probe set signals were derived using the PLIER probe set algorithm. Thresholds used for the comparisons of drug-treated versus vehicle-treated were p value ≤ 0.01, fold change ≥ 1.2.

A549 Gene Transcription Assay. A549 cells were grown to confluence in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and exposed to prednisolone (1, 10, or 100 μM), anecortave (1, 10, or 100 μM), or vehicle control (DMSO) for 4 h at 37°C and for 24 h at 37°C. Real-time polymerase chain reaction was performed for *FKBP5*, *GILZ*, *CCL2*, and *MKP-1* mRNAs, and 18S ribosomal RNA (housekeeping control) using TaqMan primers (Applied Biosystems, Foster City, CA).

Receptor Binding Assays. GR binding assays were performed by two methods. Rat liver assays were performed by the State University of New York at Buffalo using methods published previously (Almon et al., 2008). Competitive binding assays were performed by Caliper Life Sciences (Hopkinton, MA), using radiolabeled ^{3}H ligands and partially purified full-length human receptors expressed from recombinant baculovirus-infected insect cells.

Preclinical Trials. In a short-term trial, prednisolone (1 mg/kg) and anecortave (5 mg/kg) were administered orally to 8-week-old female *mdx* mice daily via syrup drops for 3 weeks. The visualization of inflammation in vivo using the noninvasive imaging of cathepsin B caged near-infrared substrate ProSense 680 (PerkinElmer, Waltham, MA) was done as described previously (Baudy et al., 2011).

ABBREVIATIONS: NF-κB, nuclear factor-κB; AR, androgen receptor; FOXO, Forkhead box O; GR, glucocorticoid receptor; GRE, glucocorticoid response element; MR, mineralocorticoid receptor; TNF-α, tumor necrosis factor-α; VBP1, anecortave desacetate; RU-486, 17β-hydroxy-11β-[4-dimethylamino phenyl]-17α-[1-propynyl]estra-4,9-dien-3-one; DMSO, dimethyl sulfoxide; HEK, human embryonic kidney.

Membrane permeability was assessed using Cy5.5-labeled 10-kDa dextran beads injected intraperitoneally (150 μl/mouse). The forelimbs and hindlimbs were scanned using the eXplore Optix (GE Medical Systems, London, ON, Canada) optical scanner 24 h after injection and then quantified using Optiview software to calculate the total number of photon counts per square millimeter of scanned area.

For the 4-month trials, 8-week-old female *mdx* mice (n = 24, 8 per group) and 12-week-old SJL mice (n = 24, 8 per group) were separated into untreated, anecortave-treated, and prednisolone-treated groups. Drugs were administered to the treated mice via food for 4 months at a dose of 40 mg/kg anecortave and 5 mg/kg prednisolone. Evaluation of function, behavior, and histology using hematoxylin and eosin of formalin-fixed, paraffin-embedded muscle was performed as described previously (Spurney et al., 2009). Force measurements were conducted on the extensor digitorum longus muscle of the right hindlimb of the *mdx* mouse as described previously (Spurney et al., 2011).

Results

Δ-9,11 Analogs Show Retention of NF-κB Transrepression Activities. To determine whether Δ-9,11 analogs retained NF-κB inhibitory activity, we used a TNF-α-induced NF-κB luciferase construct stably transfected in C_2C_{12} cells

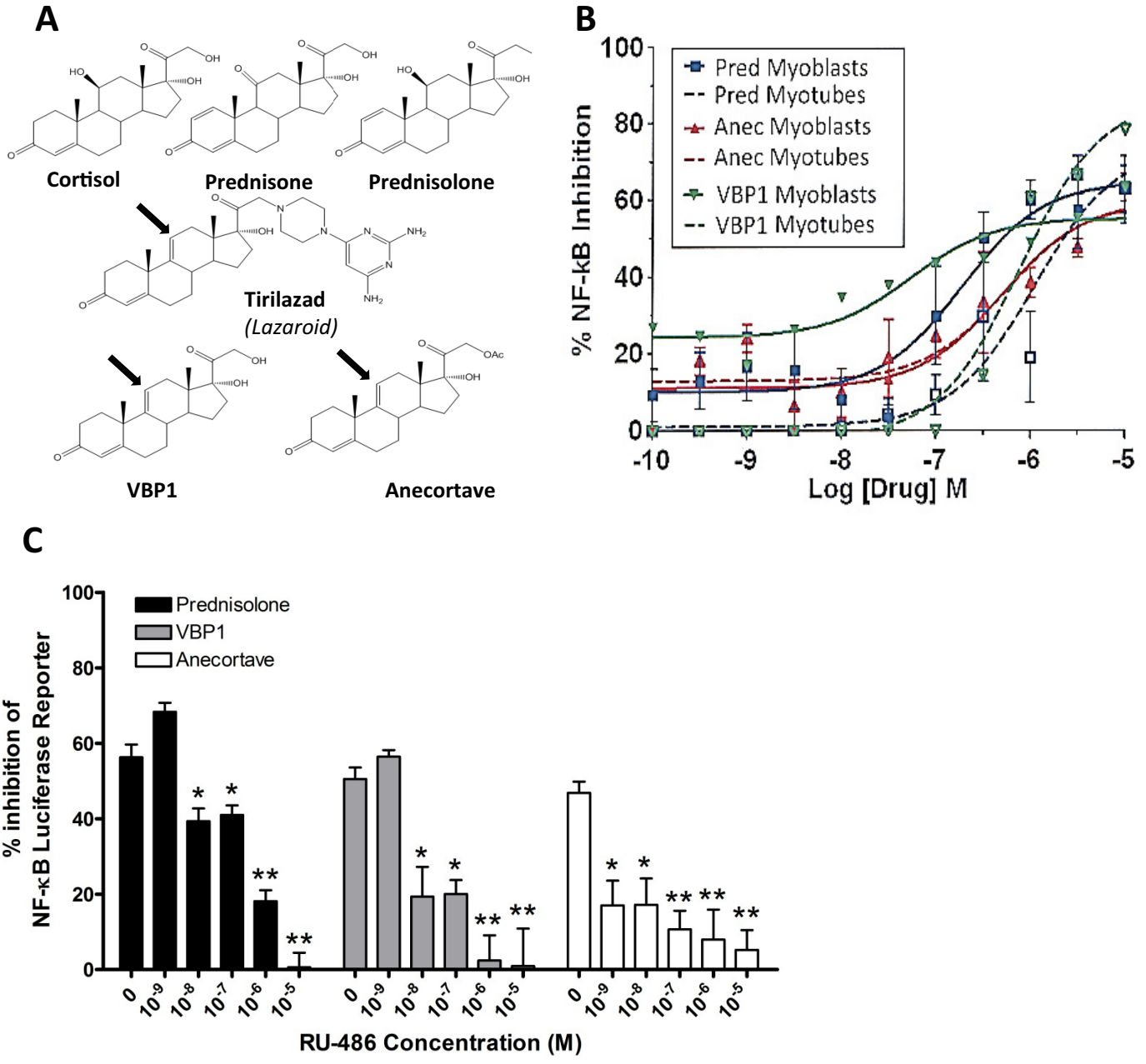


Fig. 1. Δ-9,11 Glucocorticoid analogs retain transrepression activities. A, the Δ-9,11 double bond dissociates the transactivational activities of traditional steroids (VBP1, anecortave) (arrow). Lazaroids share the Δ-9,11 double bond but have larger moieties designed for membrane integration. B, Δ-9,11 analogs show potent inhibition of TNF-α-induced NF-κB activity, similar to that seen by prednisolone. Shown is a luciferase reporter assay in C_2C_{12} cells. C, the inhibition of TNF-α-induced NF-κB activity is blocked by increasing concentrations of the GR antagonist RU-486, showing GR dependence of NF-κB inhibition (one-way analysis of variance with Dunnett's multiple comparison test: *, $p < 0.05$; **, $p < 0.01$). Mean ± S.E.M. values are representative data from three independent experiments performed in triplicate and are expressed as the percentage of reporter inhibition compared with no-drug controls.

and studied drug effects on both undifferentiated myoblasts and multinucleated myotubes (Baudy et al., 2009). We synthesized anecortave, and the 21-hydroxy desacetate form, VBP1 (Fig. 1; Supplemental Fig. 1). Δ-9,11 Analogs showed NF-κB inhibitory activity in both cell differentiation stages at potencies comparable with that of prednisolone (Fig. 1B). The transrepression activities of prednisolone, anecortave, and VBP1 were blocked by increasing the concentrations of the receptor antagonist RU-486, suggesting that the NF-κB inhibitory activity of all three compounds is mediated by GR (Fig. 1C). RU-486 also antagonizes the progesterone receptor; however, we found no detectable binding of anecortave or VBP1 with progesterone receptor over a broad concentration range (see below).

To determine whether Δ-9,11 analogs induced nuclear translocation of GR, we studied dexamethasone and the two analogs using a β-galactosidase chemiluminescence binding partner assay in a CHO cell line. Both Δ-9,11 analogs induced nuclear translocation at approximately half the levels induced by dexamethasone (Fig. 2A). In addition, immunostaining of A549 cells after the incubation with VBP1 or dexamethasone for 30 min showed nuclear translocation of GR (Fig. 2B).

Δ-9,11 Analogs Show the Loss of GRE-Mediated Transactivation Activities. The ability of the Δ-9,11 analogs to mediate transcriptional activity using GREs was studied by measuring the response of a HEK293 cell line with GRE consensus sites coupled to a luciferase reporter. Prednisolone strongly induced GRE-mediated luciferase expression in a dose-dependent manner, whereas Δ-9,11 analogs showed no activity at 200 times prednisolone concentrations (Fig. 3A). These data suggested that Δ-9,11 analogs lack transactivation (GRE-mediated transcriptional) properties.

To more broadly test global transcriptional responses to prednisolone and Δ-9,11 analogs, wild-type and dystrophin-deficient *mdx* H-2K myotubes were treated with drugs followed by mRNA profiling using Affymetrix microarrays (Fig. 3B). We have shown previously that substantial transcriptional responses to corticosteroids occur in muscle approxi-

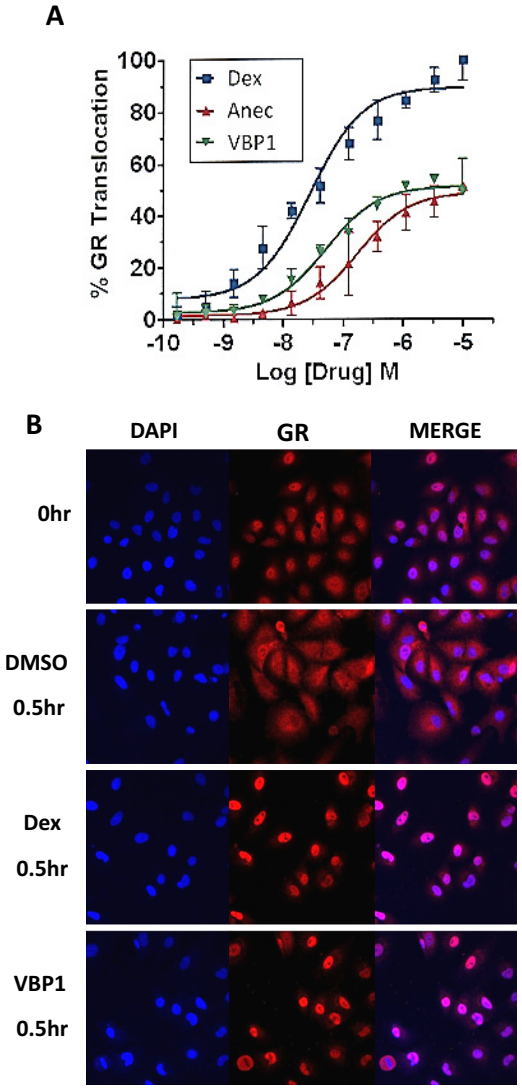


Fig. 2. Δ-9,11 Analogs induce GR translocation from the cytoplasm to nucleus. A, CHO-K1 cell line expressing β-galactosidase fragments on both GRs and a nuclear steroid coactivator peptide shows nuclear translocation induced by both glucocorticoids and Δ-9,11 analogs. B, both dexamethasone and VBP1 induce nuclear translocation of GR in A549 cells. Cells were stained for GR (red) and 4,6-diamidino-2-phenylindole (blue) for nuclear reference. A merge of the two colors is represented in the right column. Cells before exposure are shown in the top row.

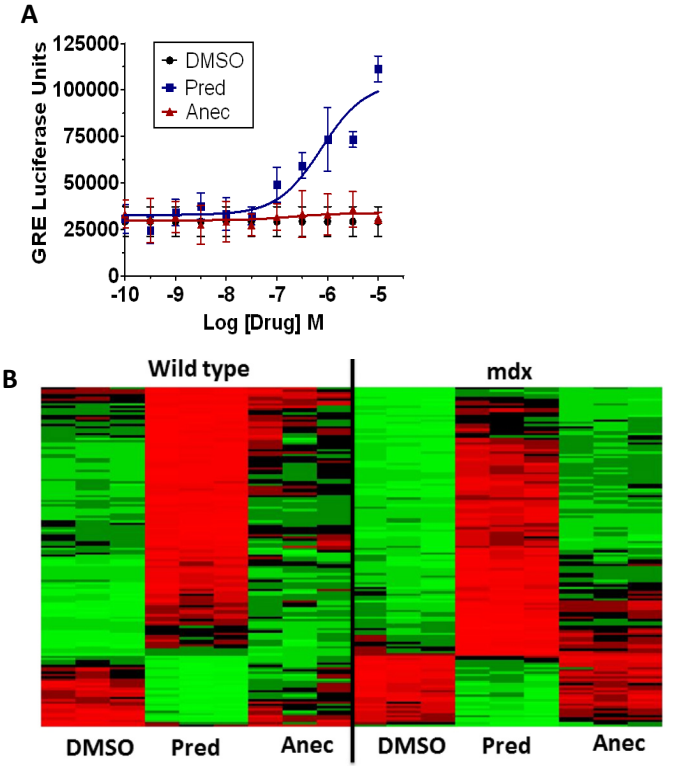


Fig. 3. Δ-9,11 Analogs show the loss of downstream GRE-mediated transactivation. A, a cell-based reporter assay with GRE promoter elements fused to luciferase shows induction by prednisolone but not anecortave, which is consistent with the loss of GRE-dependent transcription by Δ-9,11analogs. B, wild-type (WT) (n = 3) and dystrophin-deficient (*mdx*) (n = 3) H-2K myotubes were exposed to 10 μM prednisolone or anecortave for 4 h. mRNA expression profiles were generated as described under *Materials and Methods* and analyzed. Prednisolone induced a strong transcriptional response involving 148 transcripts coordinately regulated in WT and *mdx* myotubes (red = increased expression, black = no change, green = decreased expression) relative to vehicle-only controls (DMSO) ($p < 0.01$ independently for WT and *mdx*). The Δ-9,11 analog failed to induce this transcriptional pattern, appearing more similar to vehicle-only control myotubes for both wild-type and *mdx*. Selection of these genes was by statistical methods; however, the heat map does not represent statistical significance.

mately 4 to 8 h after a bolus of the drug in vivo (Almon et al., 2007; Yao et al., 2008) and selected the 4-h time point to enrich for direct transcriptional targets of ligand/receptor complexes. A total of 148 mRNA transcripts modulated by 10 μM prednisolone at 4 h in both wild-type and *mdx* cultures were seen ($p < 0.01$ and fold-change >1.2 in both experiments), with 75% transcriptionally activated by prednisolone (red color) and 25% transcriptionally repressed (green color) (Fig. 3B). The anecortave-treated cultures showed little overlap with prednisolone and instead appeared more similar to control (DMSO vehicle)-treated cultures.

The mRNA profiles were queried for molecular networks associated with GR (NRC31) (Supplemental Fig. 2A), and this showed prednisolone to cause the expected negative transcriptional regulation of GR (Burnstein et al., 1991), as well as the induction of a key muscle atrophy transcript, FOXO (Reed et al., 2012) (Supplemental Fig. 2, A and B). Comparing this to anecortave-regulated transcripts, there was little evidence of a shared transcriptional response with prednisolone, with no down-regulation of GR or transcriptional activation of FOXO1. In a different nonmuscle assay, A549 cells treated with prednisolone induced the expected transcriptional up-regulation of *FKBP5*, *GILZ*, and *MKP-1* (Chivers et al., 2006), whereas exposure to Δ-9,11 analogs did not alter the expression of these target genes (Supplemental Fig. 2C). All of the data were consistent with the loss of GRE-mediated transcriptional response by Δ-9,11 analogs.

Δ-9,11 Analogs Mediate Transrepression as Ligand/GR Monomers. To test whether GR-mediated NF-κB inhibition was a dimer- or monomer-mediated event, we used GR(dim/dim) mice transgenic for a GR gene containing a point mutation preventing dimerization (Reichardt et al., 2001). Splenocytes were isolated from GR(dim/dim) and wild-type mice, pretreated with 10 μM anecortave or prednisolone, and then tested for the inhibition of TNF-α induced NF-κB transcriptional targets using a mouse NF-κB-regulated cDNA array. We found that both prednisolone and anecortave inhibited TNF-α-induced targets efficiently in GR(dim/dim) mice but less so in wild-type mice. In addition, some genes (*IRF1*, *FASL*, *IFNB1*, *COX2*, *IFNγ*, and *IL1α*) were inhibited more strongly by anecortave than by prednisolone in wild-type splenocytes but not in GR(dim/dim) mice (Supplemental Table 1). This suggested that the NF-κB inhibitory activity was mediated by GR/ligand monomers.

Δ-9,11 Glucocorticoid Analogs Show Differential Cross-Reactivity with Nuclear Hormone Receptors. Competitive binding assays were carried out for Δ-9,11 analogs compared with known high-affinity ligands for the GR, MR, AR, and estrogen and progesterone receptors (Supplemental Fig. 3; Supplemental Table 2). VBP1 showed binding to GR at approximately 50-fold reduced affinity (triamcinolone $IC_{50} = 1.34 \times 10^{-9}$; VBP1 $IC_{50} = 6.53 \times 10^{-8}$). Neither anecortave nor VBP1 showed any detectable binding to the estrogen or progesterone receptors (Supplemental Fig. 3). For MR, binding affinity varied significantly between the different Δ-9,11 glucocorticoid analogs—VBP1 showed 11-fold higher affinity than spironolactone, whereas anecortave had a 5-fold lower binding affinity (Supplemental Fig. 3; Supplemental Table 2). For AR, anecortave and VBP1 showed >100-fold reduced affinity compared with methyltrienolone. In contrast to GR, the Δ-9,11 analogs did not induce the nuclear translocation of MR or AR (E.K.M Reeves, unpublished observations).

Given the relatively high affinities of VBP1 and anecortave for MR and the known anti-inflammatory activities of MR complexes (Yagi and Sata, 2011), we tested the abilities of prednisolone and anecortave to induce MR gene targets in normal and *mdx* myotubes. The mRNA profiles used to analyze GR targets were queried for known MR gene targets, and the data were analyzed statistically, visualized by heat maps, and tested for MR-associated networks using Ingenuity Pathway Analysis (Ingenuity Systems, Redwood City, CA). We found no evidence of the regulation of MR targets by either prednisolone or anecortave (Z. Wang and E. P. Hoffman, unpublished observations).

In Vivo Tests of Efficacy and Side Effects in Murine Models of Muscular Dystrophy. We compared anecortave to prednisolone in three preclinical trials using mouse muscular dystrophy models: one short-term, 3-week daily oral bolus dosing study to assess effects on muscle inflammation in dystrophin-deficient *mdx* mice and two longer-term, 4-month studies with administration in food in both dystrophin-deficient *mdx* and dysferlin-deficient *SJL* mice.

In the 3-week acute trial, a daily oral bolus was given in syrup (1 mg/kg per day prednisolone or 5 mg/kg per day anecortave). A significant reduction of cathepsin B activity, an indicator of muscle inflammation and regeneration, in the forelimbs of both prednisone- and anecortave-treated *mdx* mice was observed in comparison to untreated *mdx* mice as shown by the caged near-infrared cathepsin B substrate Prosense 680 (Fig. 4A) (Baudy et al., 2011).

In the 4-month trials (separate *mdx* and *SJL* trials), we used chronic dosing in food at higher levels (mouse chow prepared with ~5 mg/kg per day prednisolone or ~40 mg/kg per day anecortave) (approaching the maximum tolerated doses for both drugs). At the study end point, prednisolone-treated mice in the *mdx* mouse trial showed significant losses of both body weight and spleen weight (Fig. 4, B and C), which is consistent with previous reports of deleterious side effects in rats (Orzechowski et al., 2002), whereas anecortave at higher doses showed none of these side effects. Likewise, in the dysferlin-deficient (*SJL*) trial, prednisolone, but not anecortave, induced a significant loss of spleen weight [control, 6.09 ± 0.34 g; prednisolone, 4.11 ± 0.23 g (∗, $p < 0.05$); anecortave, 6.52 ± 0.59 g]. In the *SJL* trial, the body weights of prednisolone mice were similar to those of controls, whereas anecortave mice significantly increased body weight (control, 19.62 ± 0.62 g; prednisolone, 20.16 ± 0.41 g; anecortave, 21.83 ± 0.68 g∗). In the *SJL* trial, prednisolone induced significantly increased heart weight normalized to body weight, whereas anecortave did not induce this off-target effect (control, 4.98 ± 0.13 mg; prednisolone, 6.29 ± 0.27 mg∗; anecortave, 4.84 ± 0.20 mg).

In both *mdx* and *SJL* trials, multiple histological and functional end points reflective of drug efficacy showed improvement with both anecortave and prednisolone, and often greater efficacy was seen with anecortave. In the *mdx* trial, inflammatory infiltrates decreased, whereas muscle force increased significantly with anecortave (Supplemental Fig. 4). In the *SJL* trial, forelimb grip strength increased significantly in anecortave mice (control, 95 ± 3 g; prednisolone, 97 ± 1 g; anecortave, 108 ± 3 g∗), as did performance on a rotorod motor coordination test (control, 103.6 ± 4.76 s; pred-

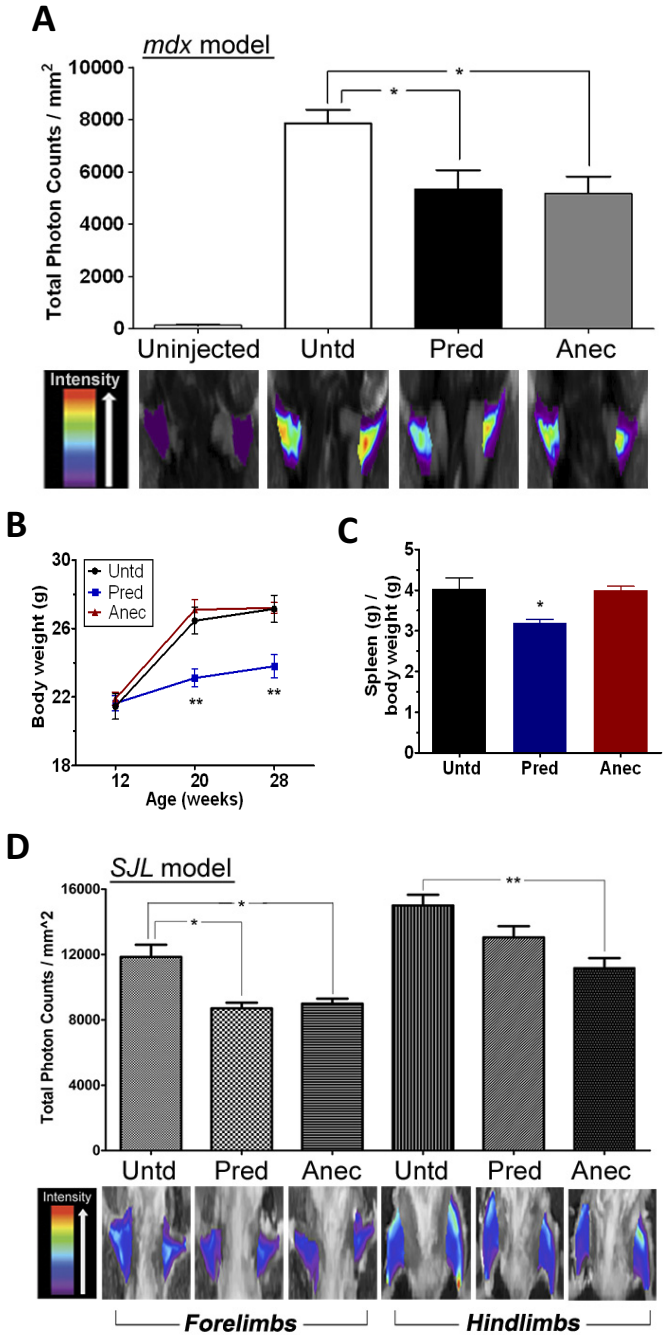


Fig. 4. A Δ-9,11 analog improves muscular dystrophy in both *mdx* and *SJL* models, with no evidence of prednisolone-associated side effects. A, cathepsin B optical imaging of the forelimbs was performed on *mdx* mice that were administered daily prednisone or anecortave syrup for 3 weeks. Both drugs showed significant reductions in cathepsin B levels, an enzyme directly relaying levels of muscle inflammation and regeneration (illustrated as a photon intensity map; purple = lowest photon counts, red = highest) (Student's unpaired *t* test comparing untreated to each drug-treated group; ∗, $p < 0.05$). B and C, a 4-month trial was conducted in *mdx* mice receiving prednisolone or anecortave in food. Prednisone-treated mice, but not Δ-9,11 analog-treated mice, showed the expected side effects of loss of body weight (B) and spleen weight (C). (One-way analysis of variance with Bonferroni multiple comparison post hoc test: ∗, $p < 0.05$; ∗∗, $p < 0.01$.) D, *SJL* (dysferlin-deficient) mice were treated with prednisolone (5 mg/kg) or anecortave (40 mg/kg) for 4 months in food. A 10-kDa dextran coupled to Cy5.5 dye was injected intraperitoneally (150 μl per mouse), and the inclusion of the dye into muscle in vivo was assessed 24 h after injection by live animal optical scanning. Anecortave-treated muscle showed improved resistance to dye inclusion, suggesting partial rescue of plasma membrane defects (Student's unpaired *t* test comparing untreated to each drug-treated group; ∗, $p < 0.05$; ∗∗, $p < 0.01$).

nisolone, 121.1 ± 4.04 s; anecortave, 137.9 ± 17.64 s∗). Histologically, there were significantly fewer central nucleated fibers in anecortave-treated mice, indicating protection from myofiber degeneration (control, 154.3 ± 12.2 central nuclei per field; prednisolone, 146.7 ± 18.4 central nuclei per field; anecortave, 111.8 ± 5.2∗ central nuclei per field). Assessments of myofiber membrane integrity using optical imaging of muscle after intraperitoneal injection of Cy5.5-labeled 10-kDa dextran beads showed significant reductions in myofiber dye uptake in both prednisolone- and anecortave-treated mouse forelimbs and hindlimbs (Fig. 4D), although the effect in hindlimbs was more pronounced for anecortave relative to that for prednisolone.

Discussion

Existing models of glucocorticoid anti-inflammatory activities involve ligand binding to the GR cytoplasmic receptor. Previous reports using transgenic mice harboring GR mutations that prevent dimerization of GR [GR(dim/dim)] have shown that these mice retain the anti-inflammatory response to pharmacological glucocorticoids but do not transactivate genes with GRE *cis*-elements (Reichardt et al., 1998). These data suggest that neither dimerization nor GRE-mediated transcriptional activation (transactivation properties) are required for the efficacy of glucocorticoids. The GR(dim/dim) data set the stage for the efforts to develop and test dissociative steroidal compounds that bind and translocate GR but do not modulate GRE-mediated transcription.

In this article, we show that a Δ-9,11 analog of glucocorticoids [anecortave (prodrug), VBP1] is a dissociative steroid—it translocates GR and shows anti-inflammatory activities yet lacks GRE-mediated transcription. To our knowledge, we present the first data testing the transrepression and transactivation activities of Δ-9,11 analogs. We found that the Δ-9,11 analog was able to bind GR (albeit at a lower affinity compared with classic ligands) and retained activity in inducing translocation of GR from the cytoplasm to the nucleus (Fig. 2) but did not induce GRE-mediated transcription. It also retained potent NF-κB inhibitory activities, at levels similar to standard GR ligands, and this transrepression activity was blocked by RU-486, showing a requirement for GR (Fig. 1C). Furthermore, this NF-κB inhibitory activity still was retained in cells unable to form GR dimers and undergo GRE-mediated transactivation [GR(dim/dim) transgenic mice], suggesting that transpression was mediated by ligand/GR monomers. The Δ-9,11 analog showed anti-inflammatory activity in vivo in acute (4 weeks) and long-term (4 month) studies in two animal models of muscular dystrophy (dystrophin-deficient *mdx* and dysferlin-deficient *SJL*). Efficacy of the Δ-9,11 analog using multiple histological and functional outcome measures was equal to or greater than that of prednisolone, although dosing regimens varied, and more extensive dose optimization is required (Fig. 4).

Many inflammatory genes are regulated by both direct GR/GRE action on their gene promoters as well as NF-κB DNA binding. Indeed, part of the complexity of GR action on anti-inflammatory genes includes GR/NF-κB antagonism at the level of gene promoters, leading to the steric occlusion of NF-κB DNA binding by GR (Novac et al., 2006). Given our model of the dissociative activity of Δ-9,11 analogs, we would expect NF-κB DNA binding to be retained but GRE-mediated DNA binding to be lost, thus leading to differential regula-

tion of the expression of genes that have both GREs and NF-κB promoter elements (*IRF1*, *FASL*, *IFNB1*, *COX2*, and *IFNγ*) versus those that have only NF-κB promoter elements (*IL-6*, *NOS2*, *MMP1*, *IL-9*, *TNFα*, *TNFR*, *MYC*, and *IL-1α*) (Supplemental Table 1). Consistent with this model, transcripts containing only NF-κB promoter elements were inhibited strongly by both prednisolone and anecortave in both wild-type and GR(dim/dim) mice. In contrast, inflammatory transcripts containing both GRE and NF-κB promoter elements were inhibited more strongly by anecortave compared with prednisolone in wild-type mice (85.5% inhibition by anecortave; 50.8% by prednisolone), and this differential activity was lost in the GR(dim/dim) mice (96% inhibition for both prednisolone and anecortave). These data suggest that Δ-9,11 analogs may prove to be more effective anti-inflammatory drugs than traditional glucocorticoids, because they remove some or all of the steric occlusion between GRE and NF-κB promoter elements.

The dissociation of transrepression from transactivation suggests that Δ-9,11 analogs have the potential for replacing current glucocorticoid drugs. Our data suggest that the analogs bind GR and induce nuclear translocation and transrepressive activities independent of dimer formation but do not permit binding to GRE elements in gene promoters (transactivation). We suggest that future optimized lead compounds could be selected based upon GR translocation and NF-κB inhibition, with reduced cross-reaction to other nuclear hormone receptors.

Acknowledgments

We thank Dr. Günther Schütz of Deutsches Krebsforschungszentrum (German Cancer Research Center) for his gift of GR(dim/dim) mice.

Authorship Contributions

Participated in research design: Baudy, Reeves, Damsker, Heier, McCall, Rose, Nagaraju, and Hoffman.

Conducted experiments: Baudy, Reeves, Damsker, Heier, Garvin, Dillingham, Rayavarapu, Wang, Vander Meulen, Sali, Jahnke, Duguez, and DuBois.

Contributed new reagents or analytic tools: McCall.

Performed data analysis: Baudy, Reeves, Damsker, and Heier.

Wrote or contributed to the writing of the manuscript: Baudy, Reeves, Damsker, Heier, McCall, Rose, Nagaraju, and Hoffman.

References

Almon RR, DuBois DC, Yao Z, Hoffman EP, Ghimbovschi S, and Jusko WJ (2007) Microarray analysis of the temporal response of skeletal muscle to methylprednisolone: comparative analysis of two dosing regimens. *Physiol Genomics* **30:** 282–299.

Almon RR, Yang E, Lai W, Androulakis IP, Ghimbovschi S, Hoffman EP, Jusko WJ, and Dubois DC (2008) Relationships between circadian rhythms and modulation of gene expression by glucocorticoids in skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* **295:**R1031–R1047.

Altavilla D, Squadrito F, Campo GM, Squadrito G, Arlotta M, Urna G, Sardella A, Quartarone C, Saitta A, and Caputi AP (1999) The lazaroid, U-74389G, inhibits inducible nitric oxide synthase activity, reverses vascular failure and protects against endotoxin shock. *Eur J Pharmacol* **369:**49–55.

Altavilla D, Squadrito F, Serrano M, Campo GM, Squadrito G, Arlotta M, Urna G, Sardella A, Saitta A, and Caputi AP (1998) Inhibition of tumour necrosis factor and reversal of endotoxin-induced shock by U-83836E, a 'second generation' lazaroid in rats. *Br J Pharmacol* **124:**1293–1299.

Bach JR, Martinez D, and Saulat B (2010) Duchenne muscular dystrophy: the effect of glucocorticoids on ventilator use and ambulation. *Am J Phys Med Rehabil* **89:**620–624.

Bajenaru ML, Piña Y, Murray TG, Cebulla CM, Feuer W, Jockovich ME, and Marin Castaño ME (2010) Gelatinase expression in retinoblastoma: modulation of LH-(BETA)T(AG) retinal tumor development by anecortave acetate. *Invest Ophthalmol Vis Sci* **51:**2860–2864.

Baschant U and Tuckermann J (2010) The role of the glucocorticoid receptor in inflammation and immunity. *J Steroid Biochem Mol Biol* **120:**69–75.

Baudy AR, Sali A, Jordan S, Kesari A, Johnston HK, Hoffman EP, and Nagaraju K (2011) Non-invasive optical imaging of muscle pathology in mdx mice using cathepsin caged near-infrared imaging. *Mol Imaging Biol* **13:**462–470.

Baudy AR, Saxena N, Gordish H, Hoffman EP, and Nagaraju K (2009) A robust in vitro screening assay to identify NF-kappaB inhibitors for inflammatory muscle diseases. *Int Immunopharmacol* **9:**1209–1214.

Bracken MB, Shepard MJ, Holford TR, Leo-Summers L, Aldrich EF, Fazl M, Fehlings M, Herr DL, Hitchon PW, Marshall LF, et al. (1997) Administration of methylprednisolone for 24 or 48 hours or tirilazad mesylate for 48 hours in the treatment of acute spinal cord injury. Results of the Third National Acute Spinal Cord Injury Randomized Controlled Trial. National Acute Spinal Cord Injury Study. *JAMA* **277:**1597–1604.

Burnstein KL, Bellingham DL, Jewell CM, Powell-Oliver FE, and Cidlowski JA (1991) Autoregulation of glucocorticoid receptor gene expression. *Steroids* **56:** 52–58.

Campo GM, Squadrito F, Campo S, Altavilla D, Avenoso A, Ferlito M, Squadrito G, and Caputi AP (1997) Antioxidant activity of U-83836E, a second generation lazaroid, during myocardial ischemia/reperfusion injury. *Free Radic Res* **27:** 577–590.

Chivers JE, Gong W, King EM, Seybold J, Mak JC, Donnelly LE, Holden NS, and Newton R (2006) Analysis of the dissociated steroid RU24858 does not exclude a role for inducible genes in the anti-inflammatory actions of glucocorticoids. *Mol Pharmacol* **70:**2084–2095.

Chrousos GA, Kattah JC, Beck RW, and Cleary PA (1993) Side effects of glucocorticoid treatment. Experience of the Optic Neuritis Treatment Trial. *JAMA* **269:** 2110–2112.

Clark AF (2007) Mechanism of action of the angiostatic cortisene anecortave acetate. *Surv Ophthalmol* **52(Suppl 1):**S26–S34.

De Bosscher K (2010) Selective glucocorticoid receptor modulators. *J Steroid Biochem Mol Biol* **120:**96–104.

Di Giovanni S, Molon A, Broccolini A, Melcon G, Mirabella M, Hoffman EP, and Servidei S (2004) Constitutive activation of MAPK cascade in acute quadriplegic myopathy. *Ann Neurol* **55:**195–206.

Dostert A and Heinzel T (2004) Negative glucocorticoid receptor response elements and their role in glucocorticoid action. *Curr Pharm Des* **10:**2807–2816.

Escolar DM, Hache LP, Clemens PR, Cnaan A, McDonald CM, Viswanathan V, Kornberg AJ, Bertorini TE, Nevo Y, Lotze T, et al. (2011) Randomized, blinded trial of weekend vs daily prednisone in Duchenne muscular dystrophy. *Neurology* **77:**444–452.

Fukuma K, Marubayashi S, Okada K, Yamada K, Kimura A, and Dohi K (1999) Effect of lazaroid U-74389G and methylprednisolone on endotoxin-induced shock in mice. *Surgery* **125:**421–430.

Harris JM, Morgan JE, Rosenblatt JD, Peckham M, Edwards YH, Partridge TA, and Porter AC (1999) Forced MyHCIIB expression following targeted genetic manipulation of conditionally immortalized muscle precursor cells. *Exp Cell Res* **253:** 523–532.

Hillier SG (2007) Diamonds are forever: the cortisone legacy. *J Endocrinol* **195:**1–6.

Hussein MR, Abu-Dief EE, Kamel NF, and Mostafa MG (2010) Steroid therapy is associated with decreased numbers of dendritic cells and fibroblasts, and increased numbers of satellite cells, in the dystrophic skeletal muscle. *J Clin Pathol* **63:**805–813.

Kavanagh RJ and Kam PC (2001) Lazaroids: efficacy and mechanism of action of the 21-aminosteroids in neuroprotection. *Br J Anaesth* **86:**110–119.

Morgan JE, Beauchamp JR, Pagel CN, Peckham M, Ataliotis P, Jat PS, Noble MD, Farmer K, and Partridge TA (1994) Myogenic cell lines derived from transgenic mice carrying a thermolabile T antigen: a model system for the derivation of tissue-specific and mutation-specific cell lines. *Dev Biol* **162:**486–498.

Newton R and Holden NS (2007) Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? *Mol Pharmacol* **72:**799–809.

Novac N, Baus D, Dostert A, and Heinzel T (2006) Competition between glucocorticoid receptor and NFkappaB for control of the human FasL promoter. *FASEB J* **20:**1074–1081.

Orzechowski A, Ostaszewski P, Wilczak J, Jank M, Bałasińska B, Wareski P, and Fuller J Jr (2002) Rats with a glucocorticoid-induced catabolic state show symptoms of oxidative stress and spleen atrophy: the effects of age and recovery. *J Vet Med A Physiol Pathol Clin Med* **49**:256–263.

Passaquin AC, Lhote P, and Rüegg UT (1998) Calcium influx inhibition by steroids and analogs in C2C12 skeletal muscle cells. *Br J Pharmacol* **124:**1751–1759.

Puthucheary Z, Harridge S, and Hart N (2010) Skeletal muscle dysfunction in critical care: wasting, weakness, and rehabilitation strategies. *Crit Care Med* **38:**S676–S682.

Reed SA, Sandesara PB, Senf SM, and Judge AR (2012) Inhibition of FoxO transcriptional activity prevents muscle fiber atrophy during cachexia and induces hypertrophy. *FASEB J* **26:**987–1000.

Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, et al. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. *Cell* **93:**531–541.

Reichardt HM, Tuckermann JP, Göttlicher M, Vujic M, Weih F, Angel P, Herrlich P, and Schütz G (2001) Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. *EMBO J* **20:**7168–7173.

Rhen T and Cidlowski JA (2005) Antiinflammatory action of glucocorticoids–new mechanisms for old drugs. *N Engl J Med* **353:**1711–1723.

Schara U, Mortier, and Mortier W (2001) Long-term steroid therapy in Duchenne muscular dystrophy-positive results versus side effects. *J Clin Neuromuscul Dis* **2:**179–183.

Spurney CF, Gordish-Dressman H, Guerron AD, Sali A, Pandey GS, Rawat R, Van Der Meulen JH, Cha HJ, Pistilli EE, Partridge TA, et al. (2009) Preclinical drug trials in the mdx mouse: assessment of reliable and sensitive outcome measures. *Muscle Nerve* **39:**591–602.

Spurney CF, Sali A, Guerron AD, Iantorno M, Yu Q, Gordish-Dressman H, Rayavarapu S, van der Meulen J, Hoffman EP, and Nagaraju K (2011) Losartan

decreases cardiac muscle fibrosis and improves cardiac function in dystrophin-deficient mdx mice. *J Cardiovasc Pharmacol Ther* **16:**87–95.
Taylor BM, Fleming WE, Benjamin CW, Wu Y, Mathews WR, and Sun FF (1996) The mechanism of cytoprotective action of lazaroids I: Inhibition of reactive oxygen species formation and lethal cell injury during periods of energy depletion. *J Pharmacol Exp Ther* **276:**1224–1231.
Yagi S and Sata M (2011) Pre-clinical data on the role of mineralocorticoid receptor antagonists in reversing vascular inflammation. *Eur Heart J Suppl* **13 (Suppl B):**B15–B20.
Yao Z, Hoffman EP, Ghimbovschi S, Dubois DC, Almon RR, and Jusko WJ (2008) Mathematical modeling of corticosteroid pharmacogenomics in rat muscle following acute and chronic methylprednisolone dosing. *Mol Pharm* **5:**328–339.

Address correspondence to: Dr. Eric P. Hoffman, Center for Genetic Medicine Research, Children's National Medical Center, 111 Michigan Ave. NW. Washington, DC 20010. E-mail: ehoffman@cnmcresearch.org

OPEN ACCESS Freely available online

PLOS ONE

VBP15, a Glucocorticoid Analogue, Is Effective at Reducing Allergic Lung Inflammation in Mice

Jesse M. Damsker[1]*, Blythe C. Dillingham[1,2], Mary C. Rose[2,3], Molly A. Balsley[4], Christopher R. Heier[2], Alan M. Watson[2], Erik J. Stemmy[4], Roslyn A. Jurjus[4], Tony Huynh[2,5], Kathleen Tatem[2], Kitipong Uaesoontrachoon[2], Dana M. Berry[2], Angela S. Benton[2], Robert J. Freishtat[2,3], Eric P. Hoffman[1,2,3], John M. McCall[1,6], Heather Gordish-Dressman[2,3], Stephanie L. Constant[4], Erica K. M. Reeves[1], Kanneboyina Nagaraju[1,2,3]

1 ReveraGen BioPharma, Rockville, Maryland, United States of America, **2** Research Center for Genetic Medicine, Children's National Medical Center, Washington DC, United States of America, **3** Department of Integrative Systems Biology, Children's National Medical Center and George Washington University School of Medicine and Health Sciences, Washington DC, United States of America, **4** Department of Microbiology, Immunology, and Tropical Medicine, the George Washington University Medical Center, Washington DC, United States of America, **5** Endocrine Research Unit and the Australian National University Medical School, The Canberra Hospital, Australian Capital Territory, Australia, **6** PharMac LLC, Boca Grande, Florida, United States of America

Abstract

Asthma is a chronic inflammatory condition of the lower respiratory tract associated with airway hyperreactivity and mucus obstruction in which a majority of cases are due to an allergic response to environmental allergens. Glucocorticoids such as prednisone have been standard treatment for many inflammatory diseases for the past 60 years. However, despite their effectiveness, long-term treatment is often limited by adverse side effects believed to be caused by glucocorticoid receptor-mediated gene transcription. This has led to the pursuit of compounds that retain the anti-inflammatory properties yet lack the adverse side effects associated with traditional glucocorticoids. We have developed a novel series of steroidal analogues (VBP compounds) that have been previously shown to maintain anti-inflammatory properties such as NFκB-inhibition without inducing glucocorticoid receptor-mediated gene transcription. This study was undertaken to determine the effectiveness of the lead compound, VBP15, in a mouse model of allergic lung inflammation. We show that VBP15 is as effective as the traditional glucocorticoid, prednisolone, at reducing three major hallmarks of lung inflammation—NFκB activity, leukocyte degranulation, and pro-inflammatory cytokine release from human bronchial epithelial cells obtained from patients with asthma. Moreover, we found that VBP15 is capable of reducing inflammation of the lung *in vivo* to an extent similar to that of prednisone. We found that prednisolone–but not VBP15 shortens the tibia in mice upon a 5 week treatment regimen suggesting effective dissociation of side effects from efficacy. These findings suggest that VBP15 may represent a potent and safer alternative to traditional glucocorticoids in the treatment of asthma and other inflammatory diseases.

Citation: Damsker JM, Dillingham BC, Rose MC, Balsley MA, Heier CR, et al. (2013) VBP15, a Glucocorticoid Analogue, Is Effective at Reducing Allergic Lung Inflammation in Mice. PLoS ONE 8(5): e63871. doi:10.1371/journal.pone.0063871

Editor: Thomas H. Thatcher, University of Rochester Medical Center, United States of America

Received January 19, 2013; **Accepted** April 11, 2013; **Published** May 7, 2013

Funding: Supported in part by grants from the NIH (1R41HL104939-01B; 1K26RR032082; 1P50AR060836-01; 1U54HD071601; 2R24HD050846-06, R01 HL033152-25), DOD grants (W81XWH-11-1-0330; W81XWH-11-1-0782; W81XWH-10-1-0659; W81XWH-11-1-0809; W81XWH-09-1-0599) a translational research grant from MDA, pilot grant from Parent Project Muscular Dystrophy (PPMD), and a contribution from the Clark Family Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: Jesse M. Damsker is an employee of ReveraGen BioPharma and has stock options. Blythe C. Dillingham was an employee of ReveraGen BioPharma during the time the work was done on this manuscript. Erica K. M. Reeves is an employee of ReveraGen BioPharma and has stock options. John M. McCall is an employee of PharMac LLC and has founder shares and a board membership with ReveraGen BioPharma. Eric P. Hoffman has founder shares and a board membership with ReveraGen BioPharma. Finally, Kanneboyina Nagaraju has founder shares and a board membership with ReveraGen BioPharma. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

* E-mail: Jesse.Damsker@reveragen.com

Introduction

Asthma is a chronic multi-factorial disorder characterized by airway inflammation, hyper-responsiveness and mucus hypersecretion, which reflects remodeling of the airway epithelium and of the underlying basal lamina [1,2]. Asthma is one of the most common airway inflammatory disorders and its incidence continues to increase in the United States [3]. The majority of asthma cases are mediated by an allergic response to environmental allergens, which results in degranulation of leukocytes such as eosinophils and mast cells [4,5,6,7,8,9], bronchoconstriction, smooth muscle contraction, airway inflammation and mucus overproduction [10]. In addition, the asthmatic epithelium is inherently inflammogenic and upon injury (e.g. tobacco smoke, viral exposure, mechanical wounding) can release pro-inflammatory cytokines in the absence of inflammatory cells [11,12,13,14,15,16].

Glucocorticoids are among the most prescribed drugs for therapeutic management of a wide variety of acute and chronic inflammatory conditions including lupus, myositis, rheumatoid arthritis, muscular dystrophy, and asthma [17,18,19,20]. Despite their effectiveness, long-term treatment with glucocorticoids is

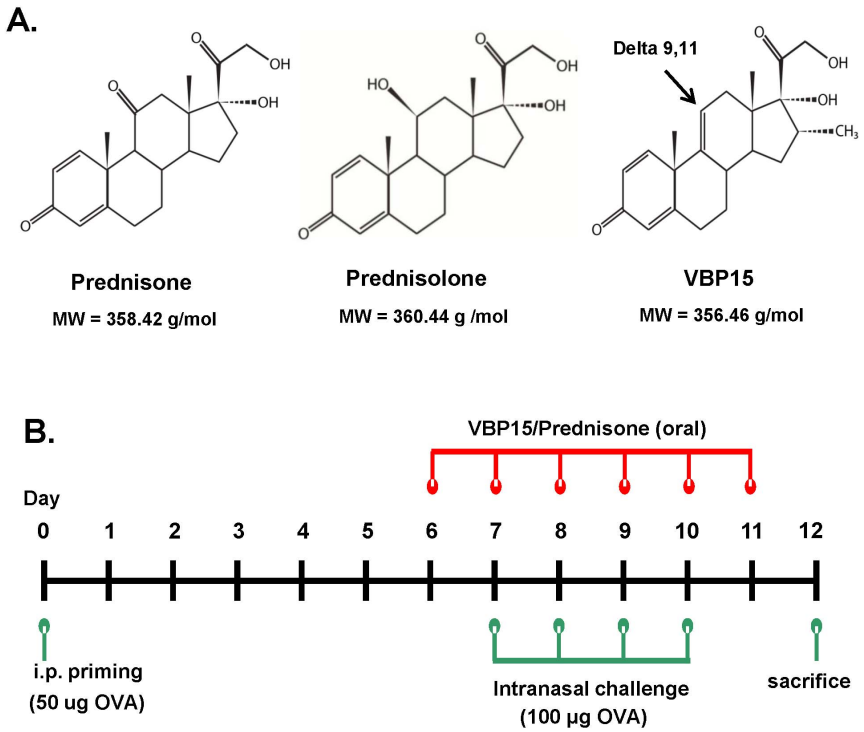


Figure 1. Structure of VBP15 and schematic of the OVA-induced model of acute allergic lung inflammation. (A) Chemical structures of prednisone (left panel), prednisolone (mid panel), and VBP15 (right panel). VBP compounds include a delta-9,11 double bond and tail group modifications. (B) Diagram of OVA-induced mouse model of allergic lung inflammation.
doi:10.1371/journal.pone.0063871.g001

limited by severe side effects including glaucoma, adrenal insufficiency, osteoporosis, cardiomyopathies, short stature, and mood or sleep disturbances [21,22,23,24,25]. In children with persistent asthma, inhaled glucocorticoids reduce growth during the first few years of therapy and height reduction persists throughout adulthood [26,27].

The beneficial anti-inflammatory properties of glucocorticoids are due to multiple mechanisms [28]. Many occur shortly (<30 minutes) after glucocorticoid exposure. For example, glucocorticoids can inhibit inflammatory transcription factors such as NFκB and AP-1 via protein-protein signaling activity, resulting in decreased production of pro-inflammatory cytokines [29,30,31,32]. Furthermore, glucocorticoids, acting via a non-genomic mechanism, can insert into cellular lipid bilayers and exert a biophysical effect on plasma membranes, affecting their structure and function, thus promoting membrane stability and even reducing cellular degranulation [33,34,35]. However, the most well-described mechanism of glucocorticoid action involves ligand binding to the cytoplasmic soluble glucocorticoid receptor (GR) to induce nuclear translocation of the ligand-receptor complex, which then interacts with glucocorticoid response elements (GREs) in the promoter regions of target genes, affecting transcription [36]. An increasing body of literature suggests that this transcriptional pathway is responsible for many of the adverse side effects associated with long-term glucocorticoid use [37,38,39].

Thus, a compound that maintains the beneficial anti-inflammatory properties of glucocorticoids but lacks the GRE-mediated transcriptional capabilities would represent an improved therapeutic option for patients suffering from inflammatory diseases. The potential for such a compound has not been well evaluated with regard to lung inflammation, although inhaled glucocorticoids contribute to systemic side-effects, and there is increasing concern about their safety for treatment of asthma in infants and young children [40,41]. Therefore, we evaluated the effectiveness of VBP15 [42], the newly-identified lead compound selected from a previously described series of Δ9,11 glucocorticoid analogues [43], in a murine model of acute allergic lung inflammation and in differentiated human bronchial epithelial (HBE) cells obtained from patients diagnosed with asthma. Additionally, we assessed the potential of long-term VBP15 treatment to inhibit bone growth *in vivo* as a means of evaluating its ability to avoid detrimental glucocorticoid-related side-effects.

Materials and Methods

Ethics Statement

All animal work was conducted according to relevant national and international guidelines.

Animals

For *in vivo* OVA-induced lung inflammation studies, female BALB/c mice at 6 weeks of age were purchased from Jackson Laboratories (Bar Harbor, Maine). For bone growth studies, timed-pregnant outbred CD-1 mice (e19) were purchased from Charles River Laboratories (Frederick, MD) and the male progeny were used for the experiment. All studies were reviewed and approved by the Institutional Animal Care and Use Committees at The George Washington University Medical Center and Children's National Medical Center.

OVA-induced Model of Acute Allergic Lung Inflammation

The lung inflammation model used in the current studies has been previously described [44,45]. Briefly, mice were primed via

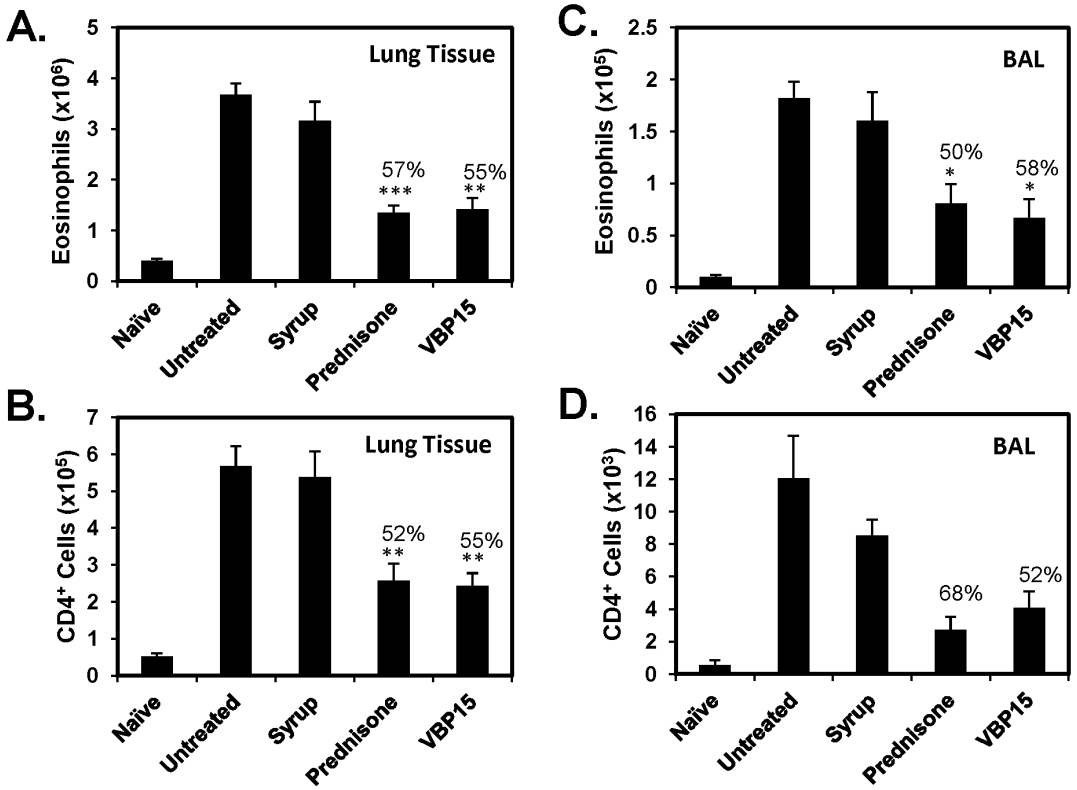


Figure 2. VBP15 reduces leukocyte infiltration in the OVA-induced model of acute allergic lung inflammation. OVA-challenged mice were either left untreated or treated with oral doses of prednisone, VBP15 (20 mg/kg), or cherry syrup alone daily for 6 days. A group of non-challenged mice (naïve) was included in order to assess basal inflammatory parameters. Lung tissue and BAL cells underwent FACS analysis to determine the number of infiltrating eosinophils (A and C) and effector/memory CD4+T cells (B and D). Bar graphs represent mean (±SE) cell numbers. Percentages indicate percentage reduction compared to vehicle control. *$p<0.05$; **$p<0.01$; ***$p<.001$ compared to syrup group with n = 5 mice per group.
doi:10.1371/journal.pone.0063871.g002

intraperitoneal (i.p.) injection with 50 µg of ovalbumin (OVA) in PBS with 100 µl of alum (200 µl total volume per mouse) on day 0. OVA-primed mice were challenged under light anesthesia (isoflurane) by intranasal delivery of 100 µg of OVA in PBS on days 7–10. Mice were sacrificed via CO_2 exposure on day 12 for analysis. For *in vivo* intervention studies, mice (n = 5) received an oral dose of 20 mg/kg VBP-15 suspended in cherry syrup (30 µl total volume) on days 6–11. Additional groups of mice (n = 5) received 5 mg/kg of prednisone in cherry syrup as a positive control, or cherry syrup alone as a negative control. Following sacrifice on day 12, leukocytes were collected from the airways via bronchoalveolar lavage (BAL). For this procedure, a cannula was inserted into the trachea and two 1 ml washes of cold PBS were infused in and out of the airways. BAL fluid from individual mice was then centrifuged and supernatants were stored at −80°C for cytokine analysis. Following BAL, whole lungs were perfused via the right ventricle with 20 ml of cold PBS. For some experiments the lungs were then removed, chopped, and pushed through a metal strainer to generate single-cell suspensions. BAL and lung tissue cells were treated with ammonium chloride lysis buffer to remove red blood cells. The remaining leukocytes were then counted and stained for FACS analysis with a combination of PE-Cy5-anti-mouse CD4 and FITC-anti-mouse CD62L to identify effector/memory $CD4^+$ cells ($CD4^+/CD62L^-$). Populations of eosinophils were identified using forward scatter/side scatter distribution, as previously described [44]. For studies addressing lung histopathology, 1 ml of 10% formalin was infused into the trachea following lung perfusion, and suture thread was used to tie off the inflated lungs. Fixed lungs were sent in 70% ethanol to Histoserv Inc. (Germantown, MD) for processing and staining with hematoxylin and eosin (H&E) and periodic acid Schiff (PAS). The frequency of PAS-positive airways was determined in a blinded manner by separately counting total airways and PAS-positive airways in each section via bright field microscopy.

NFκB Inhibition Assay

The NFκB inhibition assay was modified from a previously described protocol [46]. Briefly, A549 epithelial cells (Panomics, Fremont, CA) stably transfected with a luciferase reporter construct regulated under NFκB response elements were grown in 75 mm^2 flasks according to the manufacturer's instructions and transferred to 96-well plates upon reaching confluency. Once confluent, cells were serum starved for 48 hours and subsequently treated for 2 hours with increasing doses of VBP15, prednisolone (the active form of prednisone), or DMSO in serum-free medium at 37°C. Following treatment, cells were washed twice with PBS and exposed to TNF-α (100 ng/ml) for 6 hours in serum-free medium at 37°C. Cells were then washed once with PBS and lysed with lysis buffer (Promega Corp, Madison, WI) in order to measure luciferase activity with the Centro LB 960 luminometer (Berthold Technologies, GmbH & Co, Bad Wildbad, Germany).

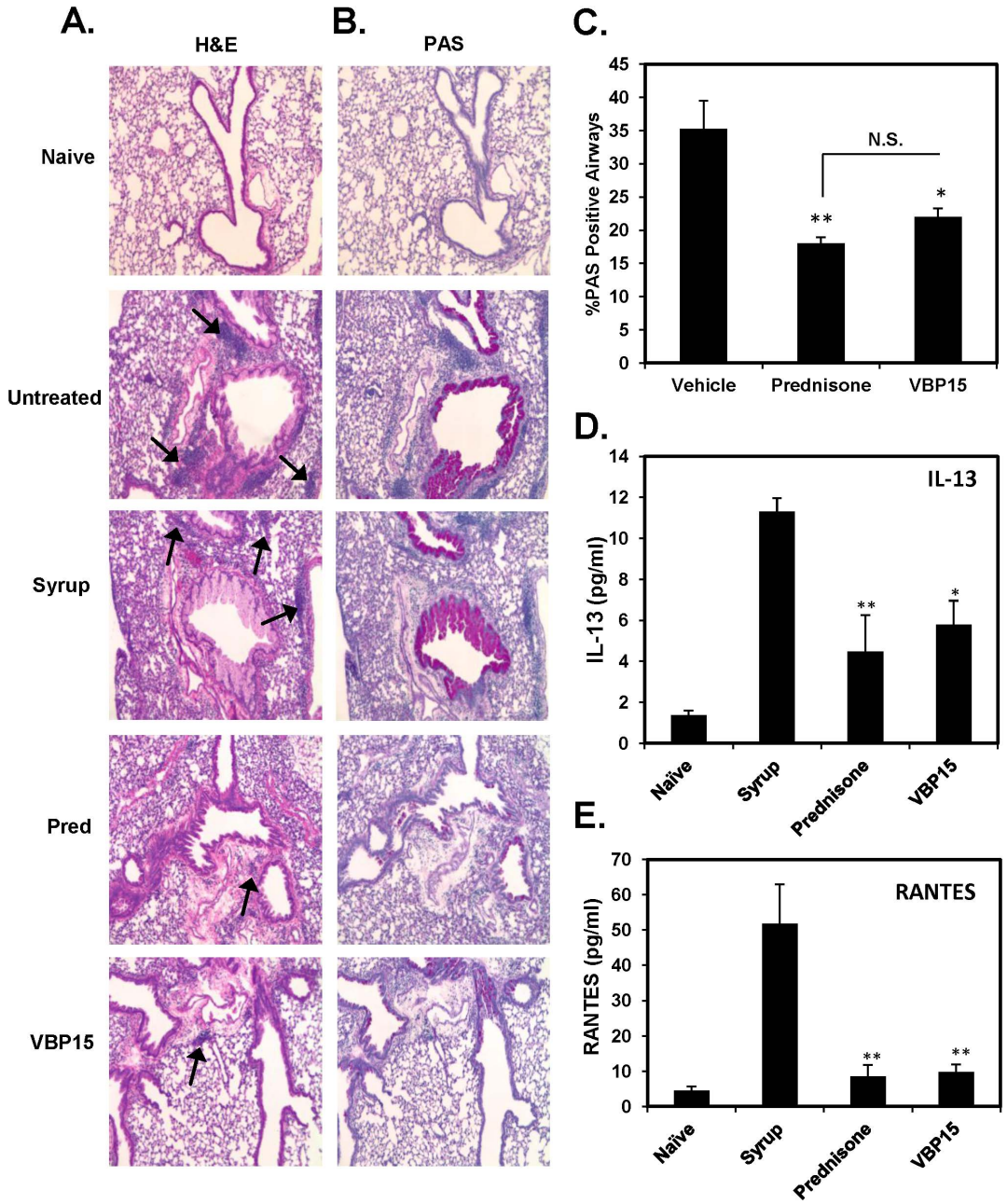


Figure 3. VBP15 reduces acute allergic lung inflammation. OVA-challenged mice were either left untreated or treated with oral doses of prednisone, VBP15 (20 mg/kg), or cherry syrup alone daily for 6 days. A group of non-challenged mice (naïve) was included in order to assess basal inflammatory parameters. Perfused whole lungs were processed for histological analysis and stained with H&E (A) or PAS (B). Images (10× magnification) represent areas of tissue surrounding bronchioles. Arrows on H&E sections indicate inflammatory foci. Percentage of PAS positive airways were counted via bright field microscopy (C). Bar graph represents mean (±SE)% PAS positive airways. *$p<.05$; **$p<0.01$ compared to the vehicle control group with n = 5 mice per group. IL-13 (D) and RANTES (E) were measured in BAL fluid by flow cytometric bead array. Bar graphs represent mean (±SE) cytokine concentration values. *$p<.05$; **$p<0.01$ compared to syrup group with n = 5 mice per group.
doi:10.1371/journal.pone.0063871.g003

Human Bronchial Epithelial Cell Culture

Primary differentiated human bronchial epithelial (HBE) cells obtained from human asthma patients (n = 3) cultured in 12-well plates on collagen-coated Transwell membrane inserts at an air-liquid interface were obtained commercially (#AIR-606-Asthma; MatTek Corporation, Ashland, MA). Upon arrival, cells were washed in PBS, placed in proprietary medium provided by the manufacturer, and cultured for 16 hours at 37°C and 5% CO_2 after which medium was replaced with identical medium lacking epidermal growth factor (EGF) and glucocorticoids. Cells were maintained under these conditions for an additional 22 hours before being pulse-treated at the basolateral surface for 2 hours with VBP-15 (10μM) or vehicle control (DMSO). Following pulsing, cells were placed in EGF/glucocorticoid-free medium, and 24 hours later, were pulse-treated again for 2 hours.

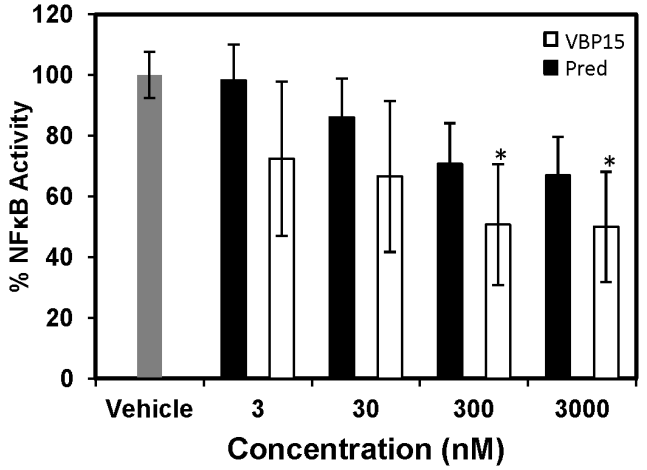


Figure 4. VBP15 inhibits NFκB activity. A549 cells stably-transfected with a luciferase NFκB construct were exposed to increasing concentrations of VBP15 or prednisolone (3, 30, 300, 3000 nM) followed by TNFα stimulation before measuring luciferase activity. Bar graph represents mean (±SE) luciferase units. *$p<.012$ (due to the Bonferroni adjustment for multiple comparisons) compared to treatment with vehicle control. Data represents 4 biological replicates with assay performed in triplicate.
doi:10.1371/journal.pone.0063871.g004

Basolateral supernatant was removed and stored at −80°C for cytokine analysis.

Measurement of Cytokines

BAL fluids from individual mice were concentrated 4-fold using 3-kDa cut-off Centricon columns (Millipore, Billerica, MA). Cytokines from BAL fluid and HBE supernatants were measured via cytometric bead assays (EBioscience, San Diego, CA) according to the manufacturer's protocol.

RBL-2H3 Degranulation Assay

β-hexosaminidase release was used as an index of degranulation. Initially, RBL-2H3 cells (ATCC, Manassas, VA) were plated in 24-well tissue culture plates in growth media consisting of Dulbecco's Modified Eagle's Medium (DMEM), 10% FBS, and 1% penicillin-streptomycin. Cells were sensitized overnight with 1 μg/ml of anti-dinitrophenol (DNP) clone SPE-7 (Sigma-Aldrich, St. Louis, MO) at 37°C in growth media. Following sensitization, cells were washed with PBS, and Earl's Balanced Salt Solution (EBSS) (Invitrogen, Carlsbad, CA)+2.5% BSA was added to each well. Cells were then treated with VBP-15 (50 μM), prednisolone (50 μM), or an equal volume of DMSO for 7 minutes at 37°C. Subsequently, DNP-BSA (Sigma-Aldrich) at a concentration of 10 μg/ml was added to each well, and cells were incubated for an additional 20 minutes at 37°C. A well of untreated cells was lysed using 0.05% Triton-X-100 as a means of gauging total β-hexosaminidase content. Supernatants and lysates were added to a 96-well plate (20 μl/well) and β-hexosaminidase substrate, p-nitrophenyl N-acetyl-beta-D-glucosamine (Sigma-Aldrich) in 0.05 M citrate buffer was added at a concentration of 1 mM in a volume of 20 μl to each well. EBSS+BSA and lysis buffer were also included on the plate as blank controls. The 96-well plate was incubated for 45 minutes at 37°C. Following incubation, 160 μl of sodium carbonate buffer (0.05 M) was added to each well to stop the reaction. The absorbance from each well was read at 405 nm using a microplate reader (Molecular Devices, Sunnyvale, CA). β-hexosaminidase release percentages were determined using the following formula: % = (Supernatant–blank)/(Total-blank) X 100. This assay was conducted in triplicate.

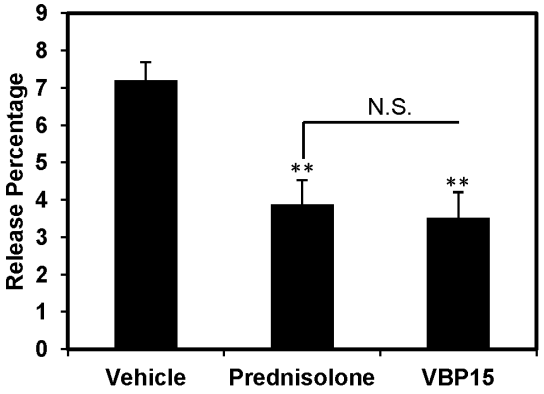


Figure 5. VBP15 reduces leukocyte degranulation. Anti-DNP-sensitized RBL-2H3 cells were treated with prednisolone (50 μM), VBP-15 (50 μM), or vehicle control (DMSO) for 7 minutes followed by addition of DNP to induce degranulation. The reaction was allowed to proceed for an additional 20 minutes before supernatant was removed and tested for β-hexosaminidase content. A well of untreated cells was lysed to gauge total β-hexosamindase content. Release percentage was determined using a formula described in Materials and Methods. Bar graph represents mean (±SE) release percentage. **$p<0.01$ compared to vehicle control. Data represents 3 biological replicates with assay performed in triplicate. N.S. = Not statistically significant.
doi:10.1371/journal.pone.0063871.g005

Assessment of in vivo bone growth

Male outbred CD-1 mice (12 days of age) were treated orally with VBP15, prednisolone, or vehicle control (cherry syrup) daily for 5 weeks after which mice were sacrificed via CO_2 and tibias were removed. Tibia lengths were measured via electronic caliper, with n = 10 mice/treatment group.

Statistical Analysis

For most experiments, statistical significance was established using one-way ANOVA with post-hoc Tukey's test. For the NFκB inhibition experiment a one-way ANOVA with Bonferroni's post-hoc test was used. For HBE cell cytokine analysis, statistical significance was established by Student's T-test. All analyses were performed using Graphpad Prism software.

Results

VBP15 reduces lung inflammation in a murine model of allergic lung inflammation

VBP compounds, in contrast to prednisone and prednisolone, contain a delta 9,11 bond (Figure 1A) that abolishes GRE-mediated gene transcription [43,47]. In the current studies we wanted to determine if our lead compound, VBP15, would retain similar anti-inflammatory efficacy compared to prednisone in the widely used OVA-induced mouse model of allergic lung inflammation (Figure 1B). After a 6-day oral dosing regimen of VBP15, we observed a significant reduction similar to that of prednisone in the number of infiltrating eosinophils (55% reduction compared to vehicle control) (Figure 2A), as well as, effector/memory CD4+T cells (55% reduction) (Figure 2B) into the lung tissue of OVA-exposed and challenged mice. These changes in the lung were also reflected in the airways as there was a striking decrease in the presence of eosinophils (58% reduction) and $CD4^+$ cells (52% reduction) in the BAL fluid (Figure 2C and D). Additionally, lung tissue sections stained with H&E to assess inflammatory pathology

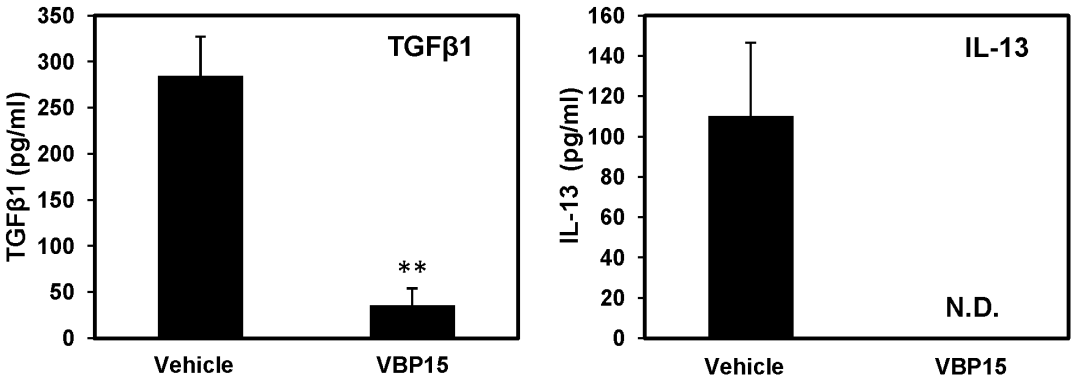


Figure 6. VBP15 reduces basolateral cytokine secretion from human bronchial epithelial cells obtained from asthmatic patients. HBE cells from 3 separate human donors were pulse-treated with VBP15 (10 μM) or vehicle control (DMSO). Basolateral surface supernatant was tested for the presence of TGFβ1 (left panel) and IL-13 (right panel) by flow cytometric bead array. Bar graphs represent mean (±SE) concentration values. **, $p<0.01$ compared to vehicle control with n = 3 donors. N.D. = Not Detectible (lower limit of detection = 4.5 pg/ml).
doi:10.1371/journal.pone.0063871.g006

and PAS to assess for mucus overproduction/hypersecretion displayed a striking reduction in the presence of inflammatory foci and PAS-positive airways in VBP15-treated mice compared to mice treated with control cherry syrup alone (Figure 3A and 3B). VBP15 treatment induced a reduction in the percentage of PAS-positive airways that was comparable to that observed in tissue sections of prednisone-treated mice (Figure 3C). Furthermore, cytokine analysis of BAL fluid from both prednisone and VBP-treated mice demonstrated significant decreases in the inflammatory cytokine, IL-13 (50% reduction), and the T-cell chemokine, RANTES (81% reduction) (Figure 3D and 3E). These findings indicate that VBP15 is capable of reducing lung inflammation *in vivo* with a similar potency to that mediated by traditional glucocorticoids.

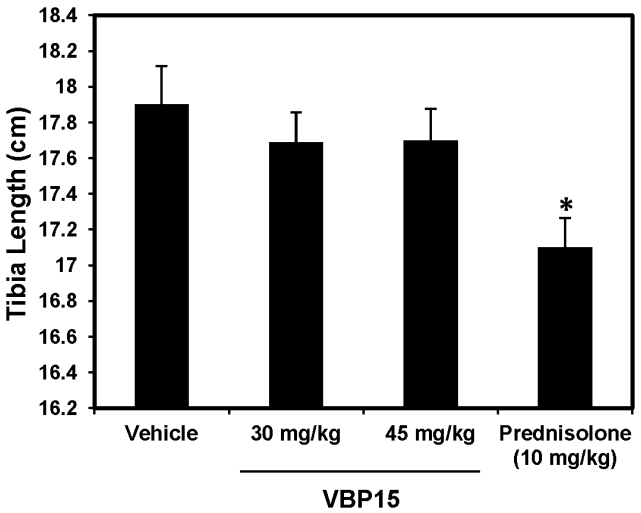


Figure 7. VBP15 does not induce tibia length shortening. Wildtype outbred CD1 mice were treated daily for 5 weeks with VBP15 (30 and 45 mg/kg), prednisolone (10 mg/kg) or vehicle control starting at 12 days of age. At the end of the treatment, tibias were harvested and measured. Bar graph represents mean (±SE) tibia length values. *$p<0.05$ compared to vehicle control with n = 10 mice/group.
doi:10.1371/journal.pone.0063871.g007

VBP15 reduces NFκB activity

Previous studies have shown that glucocorticoids can inhibit the activity of the pro-inflammatory transcription factor, NFκB, through a GRE-independent mechanism [47]. Thus, we determined whether VBP15 had the capacity to inhibit TNFα-induced NFκB expression in lung-epithelial cells to a similar extent as traditional glucocorticoids. To accomplish this, we made use of a TNF-α induced luciferase NFκB construct stably-transfected into A549 human lung epithelial cells. Interestingly, after pre-treating A549 cells with VBP15, we found that it had the capacity to reduce NFκB activity in a dose-response manner to a similar extent (56% reduction) to that observed with prednisolone, the active form of prednisone (Figure 4). Cell viability analysis via MTT assay revealed no significant differences between treatment groups (data not shown).

VBP15 reduces leukocyte degranulation

Glucocorticoids have been shown to prevent degranulation of mast cells in a guinea pig model of allergic lung inflammation via a non-genomic mechanism possibly involving stabilization of the plasma membrane [34]. We wondered if VBP15 would likewise inhibit leukocyte degranulation. We addressed this question using the RBL-2H3 cell line, which has been extensively used for studying cellular degranulation. We observed that VBP15 significantly inhibited the release of β-hexosaminidase (51% reduction), a marker for cell degranulation, to an extent similar to that mediated by prednisolone (Figure 5). This finding supports the hypothesis that VBP15 is as effective as a traditional glucocorticoid at inhibiting cell degranulation, a well-described mechanism involved in the pathogenesis of allergic lung inflammation.

VBP15 reduces inflammatory cytokine secretion from human epithelial cells

We next determined if VBP15 had an inhibitory effect on basolateral cytokine secretion in inflammatory epithelial cells from human bronchial epithelial cells (HBE) obtained from patients diagnosed with asthma, as we previously demonstrated with dexamethasone, a classical glucocorticoid [12]. After two short pulse treatments with VBP15, the basolateral secretion of TGFB1 and IL-13 from HBE cells was almost completely inhibited (87% and 100%, respectively) (Figure 6). Since epithelial cells isolated from asthma patients have been shown to release inflammatory

cytokines in the absence of immune cells, this finding suggests that VBP15 is effective at targeting non-allergy-related mechanisms as well.

VBP15 does not affect bone growth in vivo

One major known long term side-effect of glucocorticoids is the detrimental effect on bone growth. Thus, we determined whether or not extended VBP15 treatment could result in decreased bone growth by treating young mice (aged 12 days of age) daily for 5 weeks. We found that, contrary to mice treated with prednisolone, mice receiving VBP15 did not display significant tibia length shortening (Figure 7). Interestingly, we observed that doses of VBP15 four times higher than that of prednisolone did not result in any shortening of the tibia.

Discussion

Asthma is a chronic inflammatory disease that affects over 300 million people worldwide in which the majority of cases are associated with an allergic response [48]. For the past 60 years, glucocorticoids have remained the standard of care for chronic, as well as acute asthma. Despite their effectiveness, severe side effects such as osteoporosis, cardiomyopathies, and glaucoma limit long-term use [21,22,23,24,25,26,27,47,49]. Since these detrimental effects are attributed to GRE-mediated gene transcription [50], we developed a series of steroidal analogues (VBP compounds) that lack the GRE-mediated transcriptional properties of traditional glucocorticoids but retain similar anti-inflammatory potential [43]. Therefore, we hypothesized that our lead VBP compound, VBP15 [42], when benchmarked against traditional glucocorticoids, would be just as effective in terms of its ability to reduce acute allergic lung inflammation *in vivo*.

Using an acute mouse model of allergic lung inflammation, we show that VBP15 is as effective as prednisone at reducing parameters of *in vivo* inflammation including leukocyte (eosinophils and $CD4^+$ T cells) infiltration, tissue pathology, and mucus overproduction. *In vitro*, VBP15 was able to inhibit NFκB activity in lung epithelial cells to a similar degree to that seen with prednisolone (the active form of prednisone). Interestingly, we observed *in vivo* that both IL-13 and RANTES, two cytokines regulated by NFκB [51,52] that also promote respective eosinophil and $CD4^+$ T cell recruitment, were found to be reduced in the airways of VBP15 treated mice. Moreover, IL-13 is a known inducer of goblet cell metaplasia and mucus overproduction [53,54] which was strikingly decreased in lungs of mice that were treated with VBP15. A well-documented anti-inflammatory effect of traditional glucocorticoids is that they inhibit the degranulation of leukocytes via non-genomic and non-GRE-related mechanisms [34]. We observed that VBP15 was capable of reducing leukocyte cell degranulation to a similar degree to that seen with prednisolone. Since RANTES and IL-13 are two products known to be released by degranulating leukocytes [55,56] and are reduced in our *in vivo* studies, we believe that this is an additional mechanism whereby VBP15 exerts an anti-inflammatory effect within the context of acute allergic lung inflammation. It is important to note that conventional steroids may induce beneficial anti-inflammatory effects via their ability to cause GRE-mediated transcription. An example of this includes glucocorticoid-induced GRE-mediated production of IL-10 [57,58,59], a cytokine which has been shown to reduce allergic lung inflammation in mice [60]. However, despite lacking the ability to induce GRE-mediated transcription and potential IL-10 production, VBP15 still has the capacity to reduce allergic lung inflammation to a degree similar to that of conventional steroids. Mechanistic studies are currently underway addressing the effect of VBP15 on GR translocation and how it may affect a potential GR-NFκB protein interaction. Furthermore, we are planning on investigating how lung cells grown in hormone-depleted media respond to VBP15 treatment *in vitro*.

A major long term side-effect of glucocorticoids is their detrimental effect on bone growth, especially in children with asthma. Our data show that daily treatment of young mice with VBP15 at doses as high as 45 mg/kg does not inhibit bone growth of the tibia, in contrast to prednisolone. Doses of 20 mg/kg were shown to be anti-inflammatory in the OVA-induced mouse model of lung inflammation used in the current study, which mediates acute allergic responses. While similar acute responses are seen in human asthmatic patients during exacerbations, the human disease is predominantly chronic. Indeed, the acute *in vivo* model used in this study has some limitations. For example, in contrast to the human disease, airway remodeling changes accompanied by collagen deposition and fibrosis are not observed in this model. However, these changes are observed in a chronic model of allergic lung inflammation [61]. Studies to establish the potential efficacy of VBP compounds using this chronic model are currently underway. These studies will also enable us to assess *in vivo* the absence or presence of additional negative side effects traditionally seen with long-term use of glucocorticoids in chronic asthma.

The pathogenesis of asthma is complex and heterogeneous. While allergy and the immune response are known to trigger a majority of asthma cases [5,6,9], other mechanisms of pathogenesis have been described. For example, a recent study demonstrates that HBE cells of asthmatic patients possess intrinsic inflammatory properties and have the capacity to release pro-inflammatory cytokines including IL-13 and TGFβ1 from their basolateral surface without any contribution from immune cells. Interestingly, exposure of HBE cells to pulse-treatment of traditional glucocorticoids was able to significantly reduce basolateral secretion of both IL-13 and TGFβ1 [12]. We demonstrate in the current study that VBP compounds were able to almost completely eliminate basolateral secretion of IL-13 and TGFβ1 from HBE cells that were obtained from asthma patients, suggesting that these compounds may inhibit pathogenic mechanisms in addition to those associated with allergy. Ongoing work is currently in progress aimed at further characterizing the function of these cells in response to VBP15 treatment. A bulk of the non-steroidal treatment options in the clinic revolves around targeting the allergic response as a means of treating asthma (e.g. mast cell stabilizers)[62,63]. Since VBP15 may treat multiple mechanisms of asthma, and since they potentially lack the adverse glucocorticoid side effects, we believe that this compound may represent an efficacious and safer alternative to traditional glucocorticoids in the treatment of asthma and other inflammatory diseases.

Acknowledgments

The authors would like to thank Dallon Herzog and Dr. Andreas Baudy for their contributions to this study.

Author Contributions

Conceived and designed the experiments: JMD SLC RJF KN. Performed the experiments: JMD BCD MAB CRH AMW EJS RAJ TH KT KU DMB ASB. Analyzed the data: JMD BCD SLC HGD KN. Contributed reagents/materials/analysis tools: EKMR RJF SLC KN. Wrote the paper: JMD MCR.

References

1. Busse W, Banks-Schlegel S, Noel P, Ortega H, Taggart V, et al. (2004) Future research directions in asthma: an NHLBI Working Group report. Am J Respir Crit Care Med 170: 683–690.
2. Busse W, Elias J, Sheppard D, Banks-Schlegel S (1999) Airway remodeling and repair. Am J Respir Crit Care Med 160: 1035–1042.
3. Akinbami LJ, Moorman JE, Liu X (2011) Asthma prevalence, health care use, and mortality: United States, 2005–2009. Natl Health Stat Report: 1–14.
4. Larche M, Robinson DS, Kay AB (2003) The role of T lymphocytes in the pathogenesis of asthma. J Allergy Clin Immunol 111: 450–463; quiz 464.
5. Cohn L, Elias JA, Chupp GL (2004) Asthma: mechanisms of disease persistence and progression. Annu Rev Immunol 22: 789–815.
6. Mayr SI, Zuberi RI, Zhang M, de Sousa-Hitzler J, Ngo K, et al. (2002) IgE-dependent mast cell activation potentiates airway responses in murine asthma models. J Immunol 169: 2061–2068.
7. Holgate ST, Robinson C, Church MK (1988) The contribution of mast cell mediators to acute allergic reactions in human skin and airways. Allergy 43 Suppl 5: 22–31.
8. Galli SJ, Costa JJ (1995) Mast-cell-leukocyte cytokine cascades in allergic inflammation. Allergy 50: 851–862.
9. Robinson DS, Hamid Q, Ying S, Tsicopoulos A, Barkans J, et al. (1992) Predominant TH2-like bronchoalveolar T-lymphocyte population in atopic asthma. N Engl J Med 326: 298–304.
10. Wills-Karp M (1999) Immunologic basis of antigen-induced airway hyperresponsiveness. Annu Rev Immunol 17: 255–281.
11. Freishtat RJ, Nagaraju K, Jusko W, Hoffman EP (2010) Glucocorticoid efficacy in asthma: is improved tissue remodeling upstream of anti-inflammation. J Investig Med 58: 19–22.
12. Freishtat RJ, Watson AM, Benton AS, Iqbal SF, Pillai DK, et al. (2011) Asthmatic Airway Epithelium is Intrinsically Inflammatory and Mitotically Dyssynchronous. Am J Respir Cell Mol Biol.
13. Kicic A, Sutanto EN, Stevens PT, Knight DA, Stick SM (2006) Intrinsic biochemical and functional differences in bronchial epithelial cells of children with asthma. Am J Respir Crit Care Med 174: 1110–1118.
14. Holgate ST, Lackie PM, Davies DE, Roche WR, Walls AF (1999) The bronchial epithelium as a key regulator of airway inflammation and remodelling in asthma. Clin Exp Allergy 29 Suppl 2: 90–95.
15. Davies DE, Holgate ST (2002) Asthma: the importance of epithelial mesenchymal communication in pathogenesis. Inflammation and the airway epithelium in asthma. Int J Biochem Cell Biol 34: 1520–1526.
16. Hackett TL, Singhera GK, Shaheen F, Hayden P, Jackson GR, et al. (2011) Intrinsic phenotypic differences of asthmatic epithelium and its inflammatory responses to respiratory syncytial virus and air pollution. Am J Respir Cell Mol Biol 45: 1090–1100.
17. Tuckermann J, Bourguet W, Mandrup S (2010) Meeting report: nuclear receptors: transcription factors and drug targets connecting basic research with translational medicine. Mol Endocrinol 24: 1311–1321.
18. Hillier SG (2007) Diamonds are forever: the cortisone legacy. J Endocrinol 195: 1–6.
19. Larj MJ, Bleecker ER (2004) Therapeutic responses in asthma and COPD. Corticosteroids. Chest 126: 138S–149S; discussion 159S–161S.
20. (2007) Expert Panel Report 3 (EPR-3): Guidelines for the Diagnosis and Management of Asthma-Summary Report 2007. J Allergy Clin Immunol 120: S94–138.
21. Garbe E, LeLorier J, Boivin JF, Suissa S (1997) Inhaled and nasal glucocorticoids and the risks of ocular hypertension or open-angle glaucoma. JAMA 277: 722–727.
22. Wordinger RJ, Clark AF (1999) Effects of glucocorticoids on the trabecular meshwork: towards a better understanding of glaucoma. Prog Retin Eye Res 18: 629–667.
23. Manzur AY, Kuntzer T, Pike M, Swan A (2008) Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev: CD003725.
24. Rehman Q, Lane NE (2003) Effect of glucocorticoids on bone density. Med Pediatr Oncol 41: 212–216.
25. Ito T, Murata M, Kamiyama A (1979) Experimental study of cardiomyopathy induced by glucocorticoids. Jpn Circ J 43: 1043–1047.
26. Sharek PJ, Bergman DA (2000) The effect of inhaled steroids on the linear growth of children with asthma: a meta-analysis. Pediatrics 106: E8.
27. Kelly HW, Sternberg AL, Lescher R, Fuhlbrigge AL, Williams P, et al. (2012) Effect of inhaled glucocorticoids in childhood on adult height. N Engl J Med 367: 904–912.
28. Rhen T, Cidlowski JA (2005) Antiinflammatory action of glucocorticoids-new mechanisms for old drugs. N Engl J Med 353: 1711–1723.
29. Herrlich P (2001) Cross-talk between glucocorticoid receptor and AP-1. Oncogene 20: 2465–2475.
30. Coghlan MJ, Elmore SW, Kym PR, Kort ME (2003) The pursuit of differentiated ligands for the glucocorticoid receptor. Curr Top Med Chem 3: 1617–1635.
31. Baudy AR, Saxena N, Gordish H, Hoffman EP, Nagaraju K (2009) A robust in vitro screening assay to identify NF-kappaB inhibitors for inflammatory muscle diseases. Int Immunopharmacol 9: 1209–1214.
32. van der Burg B, Liden J, Okret S, Delaunay F, Wissink S, et al. (1997) Nuclear factor-kappa B repression in antiinflammation and immunosuppression by glucocorticoids. Trends Endocrinol Metab 8: 152–157.
33. Epps DE, McCall JM (1997) Physical and Chemical Mechanisms of the Antioxidant Action of Tirilazad Mesylate. In: Packer L, Cadenas E, editors. Handbook of Novel Antioxidants. New York: Marcel Dekker. pp. 95–137.
34. Zhou J, Liu DF, Liu C, Kang ZM, Shen XH, et al. (2008) Glucocorticoids inhibit degranulation of mast cells in allergic asthma via nongenomic mechanism. Allergy 63: 1177–1185.
35. Liu L, Wang YX, Zhou J, Long F, Sun HW, et al. (2005) Rapid non-genomic inhibitory effects of glucocorticoids on human neutrophil degranulation. Inflamm Res 54: 37–41.
36. Truss M, Beato M (1993) Steroid hormone receptors: interaction with deoxyribonucleic acid and transcription factors. Endocr Rev 14: 459–479.
37. Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, et al. (1998) DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93: 531–541.
38. Reichardt HM, Tuckermann JP, Gottlicher M, Vujic M, Weih F, et al. (2001) Repression of inflammatory responses in the absence of DNA binding by the glucocorticoid receptor. EMBO J 20: 7168–7173.
39. Newton R, Holden NS (2007) Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? Mol Pharmacol 72: 799–809.
40. Adcock IM, Caramori G, Chung KF (2008) New targets for drug development in asthma. Lancet 372: 1073–1087.
41. Castro-Rodriguez JA, Pedersen S (2013) The role of inhaled corticosteroids in management of asthma in infants and preschoolers. Curr Opin Pulm Med 19: 54–59.
42. Reeves EK, Hoffman EP, Nagaraju K, Damsker JM, McCall JM (2013) VBP15: Preclinical characterization of a novel anti-inflammatory delta 9,11 steroid. Bioorg Med Chem 21: 2241–2249.
43. Baudy AR, Reeves EK, Damsker JM, Heier C, Garvin LM, et al. (2012) Delta-9,11 modification of glucocorticoids dissociates nuclear factor-kappaB inhibitory efficacy from glucocorticoid response element-associated side effects. J Pharmacol Exp Ther 343: 225–232.
44. Balsley MA, Malesevic M, Stemmy EJ, Gigley J, Jurjus RA, et al. (2010) A cell-impermeable cyclosporine A derivative reduces pathology in a mouse model of allergic lung inflammation. J Immunol 185: 7663–7670.
45. Gwinn WM, Damsker JM, Falahati R, Okwumabua I, Kelly-Welch A, et al. (2006) Novel approach to inhibit asthma-mediated lung inflammation using anti-CD147 intervention. J Immunol 177: 4870–4879.
46. King EM, Holden NS, Gong W, Rider CF, Newton R (2009) Inhibition of NF-kappaB-dependent transcription by MKP-1: transcriptional repression by glucocorticoids occurring via p38 MAPK. J Biol Chem 284: 26803–26815.
47. De Bosscher K (2009) Selective Glucocorticoid Receptor modulators. J Steroid Biochem Mol Biol 120: 96–104.
48. Dougherty RH, Fahy JV (2009) Acute exacerbations of asthma: epidemiology, biology and the exacerbation-prone phenotype. Clin Exp Allergy 39: 193–202.
49. Chrousos GA, Kattah JC, Beck RW, Cleary PA (1993) Side effects of glucocorticoid treatment. Experience of the Optic Neuritis Treatment Trial. JAMA 269: 2110–2112.
50. Stahn C, Lowenberg M, Hommes DW, Buttgereit F (2007) Molecular mechanisms of glucocorticoid action and selective glucocorticoid receptor agonists. Mol Cell Endocrinol 275: 71–78.
51. Hinz M, Lemke P, Anagnostopoulos I, Hacker C, Krappmann D, et al. (2002) Nuclear factor kappaB-dependent gene expression profiling of Hodgkin's disease tumor cells, pathogenetic significance, and link to constitutive signal transducer and activator of transcription 5a activity. J Exp Med 196: 605–617.
52. Moriuchi H, Moriuchi M, Fauci AS (1997) Nuclear factor-kappa B potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J Immunol 158: 3483–3491.
53. Wills-Karp M, Luyimbazi J, Xu X, Schofield B, Neben TY, et al. (1998) Interleukin-13: central mediator of allergic asthma. Science 282: 2258–2261.
54. Grunig G, Warnock M, Wakil AE, Venkayya R, Brombacher F, et al. (1998) Requirement for IL-13 independently of IL-4 in experimental asthma. Science 282: 2261–2263.
55. Ying S, Meng Q, Taborda-Barata L, Corrigan CJ, Barkans J, et al. (1996) Human eosinophils express messenger RNA encoding RANTES and store and release biologically active RANTES protein. Eur J Immunol 26: 70–76.
56. Gessner A, Mohrs K, Mohrs M (2005) Mast cells, basophils, and eosinophils acquire constitutive IL-4 and IL-13 transcripts during lineage differentiation that are sufficient for rapid cytokine production. J Immunol 174: 1063–1072.
57. Kube D, Platzer C, von Knethen A, Straub H, Bohlen H, et al. (1995) Isolation of the human interleukin 10 promoter. Characterization of the promoter activity in Burkitt's lymphoma cell lines. Cytokine 7: 1–7.
58. Eskdale J, Kube D, Tesch H, Gallagher G (1997) Mapping of the human IL10 gene and further characterization of the 5′ flanking sequence. Immunogenetics 46: 120–128.
59. John M, Lim S, Seybold J, Jose P, Robichaud A, et al. (1998) Inhaled corticosteroids increase interleukin-10 but reduce macrophage inflammatory protein-1alpha, granulocyte-macrophage colony-stimulating factor, and inter-

feron-gamma release from alveolar macrophages in asthma. Am J Respir Crit Care Med 157: 256–262.
60. Campbell JD, Buckland KF, McMillan SJ, Kearley J, Oldfield WL, et al. (2009) Peptide immunotherapy in allergic asthma generates IL-10-dependent immunological tolerance associated with linked epitope suppression. J Exp Med 206: 1535–1547.
61. McMillan SJ, Lloyd CM (2004) Prolonged allergen challenge in mice leads to persistent airway remodelling. Clin Exp Allergy 34: 497–507.
62. Kabra SK, Lodha R (2003) Long-term management of asthma. Indian J Pediatr 70: 63–72.
63. Renzi PM (1999) Antileukotriene agents in asthma: the dart that kills the elephant? CMAJ 160: 217–223.

OPEN ACCESS
EMBO Molecular Medicine

VBP15, a novel anti-inflammatory and membrane-stabilizer, improves muscular dystrophy without side effects

*Christopher R. Heier[1], Jesse M. Damsker[2], Qing Yu[1], Blythe C. Dillingham[1,2], Tony Huynh[1,3], Jack H. Van der Meulen[1], Arpana Sali[1], Brittany K. Miller[1], Aditi Phadke[1], Luana Scheffer[1], James Quinn[1], Kathleen Tatem[1], Sarah Jordan[1], Sherry Dadgar[1,7], Olga C. Rodriguez[4,5], Chris Albanese[4,5], Michael Calhoun[6], Heather Gordish-Dressman[1,7], Jyoti K. Jaiswal[1,7], Edward M. Connor[1,2,8], John M. McCall[2,9], Eric P. Hoffman[1,2,7], Erica K. M. Reeves[2], Kanneboyina Nagaraju[1,2,7]****

Keywords: anti-inflammatory; dystrophy; *mdx*; membrane injury; muscle

DOI 10.1002/emmm.201302621

Received February 08, 2013
Revised July 30, 2013
Accepted August 02, 2013

Absence of dystrophin makes skeletal muscle more susceptible to injury, resulting in breaches of the plasma membrane and chronic inflammation in Duchenne muscular dystrophy (DMD). Current management by glucocorticoids has unclear molecular benefits and harsh side effects. It is uncertain whether therapies that avoid hormonal stunting of growth and development, and/or immunosuppression, would be more or less beneficial. Here, we discover an oral drug with mechanisms that provide efficacy through anti-inflammatory signaling and membrane-stabilizing pathways, independent of hormonal or immunosuppressive effects. We find VBP15 protects and promotes efficient repair of skeletal muscle cells upon laser injury, in opposition to prednisolone. Potent inhibition of NF-κB is mediated through protein interactions of the glucocorticoid receptor, however VBP15 shows significantly reduced hormonal receptor transcriptional activity. The translation of these drug mechanisms into DMD model mice improves muscle strength, live-imaging and pathology through both preventive and post-onset intervention regimens. These data demonstrate successful improvement of dystrophy independent of hormonal, growth, or immunosuppressive effects, indicating VBP15 merits clinical investigation for DMD and would benefit other chronic inflammatory diseases.

(1) Center for Genetic Medicine Research, Children's National Medical Center, Washington, DC, USA
(2) ReveraGen BioPharma, Rockville, MD, USA
(3) Endocrine Research Unit, The Canberra Hospital, Garran, ACT, Australia
(4) Lombardi Comprehensive Cancer Center and Department of Oncology, Georgetown University Medical Center, Washington, DC, USA
(5) Department of Pathology, Georgetown University Medical Center, Washington, DC, USA
(6) Sinq Systems, Silver Spring, MD, USA
(7) Department of Integrative Systems Biology, George Washington University School of Medicine and Health Sciences, Washington, DC, USA
(8) Center for Translational Science, Children's National Medical Center, Washington, DC, USA
(9) PharMac LLC, Boca Grande, FL, USA
***Corresponding author:** Tel: +1 202 476 6220; Fax: +1 202 476 6014; E-mail: knagaraju@childrensnational.org

INTRODUCTION

Contraction-induced myofibre injury and inflammation are characteristic features of Duchenne muscular dystrophy (DMD), a fatal genetic muscle disease. We and others have demonstrated that the pro-inflammatory transcription factor NF-κB is active in dystrophin deficient muscle before symptom onset (Chen et al, 2005; Porter et al, 2002, 2003). Pharmacological glucocorticoids (prednisone, deflazacort) are standard of care in DMD, and we hypothesize their primary mechanism of action to be through anti-inflammatory activities via NF-κB pathways (Wissink et al, 1997). However, their harsh side effects in children greatly reduce patient adherence to glucocorticoid regimens and limit their therapeutic window. More general

immunosuppressive compounds reduce inflammation in DMD but fail to increase patient strength in the same manner as glucocorticoids (Griggs et al, 1993; Kissel et al, 1993), while specific targeting of NF-κB increases strength in animal models (Grounds & Torrisi, 2004; Peterson et al, 2011). These data suggest that the specific mechanism by which glucocorticoids inhibit NF-κB is of particular importance to DMD treatment efficacy. Therapeutics that target this pathway in the absence of side effects may provide a substantial improvement in the treatment of DMD.

At the cellular level, dystrophin deficient muscles show increased susceptibility to stretch-mediated membrane instability and calcium dependent hyper-contracture (Bertorini et al, 1982; Yasuda et al, 2005), as well as increased oxidative stress (Disatnik et al, 1998; Rando et al, 1998). Glucocorticoids and other steroidal compounds are multi-mechanistic; in addition to binding hormonal receptors they can interact with the plasma membrane to exert rapid and specific physico-chemical effects (Buttgereit et al, 1999; Lipworth, 2000; Rhen et al, 2003; Shivaji & Jagannadham, 1992). These effects can alter membrane fluidity, vesicular fusion (Shivaji & Jagannadham, 1992) and ionic flux (Buttgereit et al, 1999), which are important for resistance to and repair of membrane injury. Recently, membrane stabilizing compounds such as poloxamer 188 (Spurney et al, 2011; Townsend et al, 2010), Mitsugumin 53 (Burkin & Wuebbles, 2012; Weisleder et al, 2012) and cromolyn sodium (Granchelli et al, 1996; Marques et al, 2008) have shown improvements to pathology, myofibre tension and cardiopulmonary function in dystrophin-deficient mice and dogs. The effects of glucocorticoids on membrane stability, however, have not been reported.

Because glucocorticoids act through multiple mechanisms, it has been unclear and controversial which molecular pathways provide efficacy in DMD and which are simply responsible for detrimental effects. For example, impaired growth is a glucocorticoid side effect for children with asthma (Avioli, 1993; Wolthers & Pedersen, 1990), but has been proposed as a pathway of efficacy in DMD by limiting muscle workload and delaying muscle maturation (Grounds & Shavlakadze, 2011). Further, immunotoxic effects contribute to reduced chronic inflammation, but recent evidence suggests ant-inflammatory NF-κB inhibition may be sufficient for efficacy (Peterson et al, 2011). It is clear, however, that detrimental effects of glucocorticoids currently limit their application; in DMD neonatal screening is not performed, and glucocorticoid regimens are delayed years until after the onset of fairly advanced symptoms. In other forms of muscular dystrophy, glucocorticoids are avoided altogether because the net balance of positive and negative effects is unclear. By investigating the molecular mechanisms of glucocorticoids, we have developed VBP15 as a novel oral drug. This compound is optimized for NF-κB inhibition, membrane insertion and glucocorticoid receptor (GR) specificity. Medicinal chemistry, however, both eliminates key glucocorticoid pathways and provides novel properties. Here, we present the discovery and mechanisms of this drug, then extensively examine efficacy and side effects in *mdx* muscular dystrophy model mice. We find VBP15 has novel membrane-stabilizing and immunological properties, and shows potent NF-κB inhibition and substantially reduced hormonal effects. To capitalize on this mechanism profile, which targets multiple pre-symptomatic defects, we adopt a prophylactic regimen, beginning dosing before *mdx* symptom onset in a blinded pre-clinical trial. This strategy would be analogous to a neonatal screening, preventive regimen in the clinic. Another intervention experiment in post-onset adult *mdx* mice shows repeatable efficacy in a different stage of disease. We find dose–response improvements with successful ablation of growth, bone and immunological toxicities seen with traditional glucocorticoids. These data provide new insights into biological mechanisms of efficacy *versus* side effects in DMD, identify VBP15 as a novel entity that warrants clinical investigation for DMD, and show therapeutic potential for other disorders of chronic inflammation and membrane instability.

RESULTS

In vitro characterization of VBP15

VBP15 was selected as our lead compound for clinical development from a screening program focused on Δ-9,11 compounds. This Δ-9,11 class is differentiated from glucocorticoids by the key conversion of a hydroxyl group to a carbon-carbon double bond (Fig 1). Preliminary studies suggested these drugs had potential anti-inflammatory effects (Baudy et al, 2012) but lacked activation of a synthetic GR reporter. Through extensive medicinal chemistry probing the R_1–R_3 groups of the D-ring in this steroidal structure to generate a compound library, followed by multiple lines of screening studies focused on 20 candidates, VBP15 was subsequently identified as our lead compound. Selection was based upon its superior profile in an *in vitro* assay for NF-κB inhibition in myogenic cells, in addition to ligand-induced nuclear translocation of the GR, cytotoxicity, metabolite and pharmacokinetic properties (Reeves et al, 2013). To further screen candidate compounds for target receptor specificity, we performed competitive nuclear hormone receptor binding assays (Fig 1E–H). In these assays, we found that VBP15 shows increased specificity for GR binding in comparison to other Δ-9,11 compounds. For example, VBP15 exhibited an approximately 50-fold greater affinity for the GR than VBP3, and a 64-fold lower affinity for the mineralocorticoid receptor (MR). VBP15 also showed only very low affinity for the androgen receptor (Fig 1G), over 500-fold lower than the control methyltrienolone, and lacked any detectable binding to the oestrogen (Fig 1H) or progesterone (data not shown) receptors in these *in vitro* assays. From these screening, biochemical and specificity data, VBP15 presented a superior profile for therapeutic development.

Our studies here are benchmarked against prednisolone, the active form of prednisone. Both VBP15 and prednisolone inhibited TNFα-induced pro-inflammatory NF-κB signaling at similar levels in NF-κB reporter assays in C2C12 muscle cells at 1 nM or more (Fig 2A). To confirm effects on NF-κB target genes, several inflammatory transcripts known to be induced by TNFα were assayed by qPCR in VBP15- and prednisolone-treated H2K myotubes. We found VBP15 inhibited the TNFα-induced

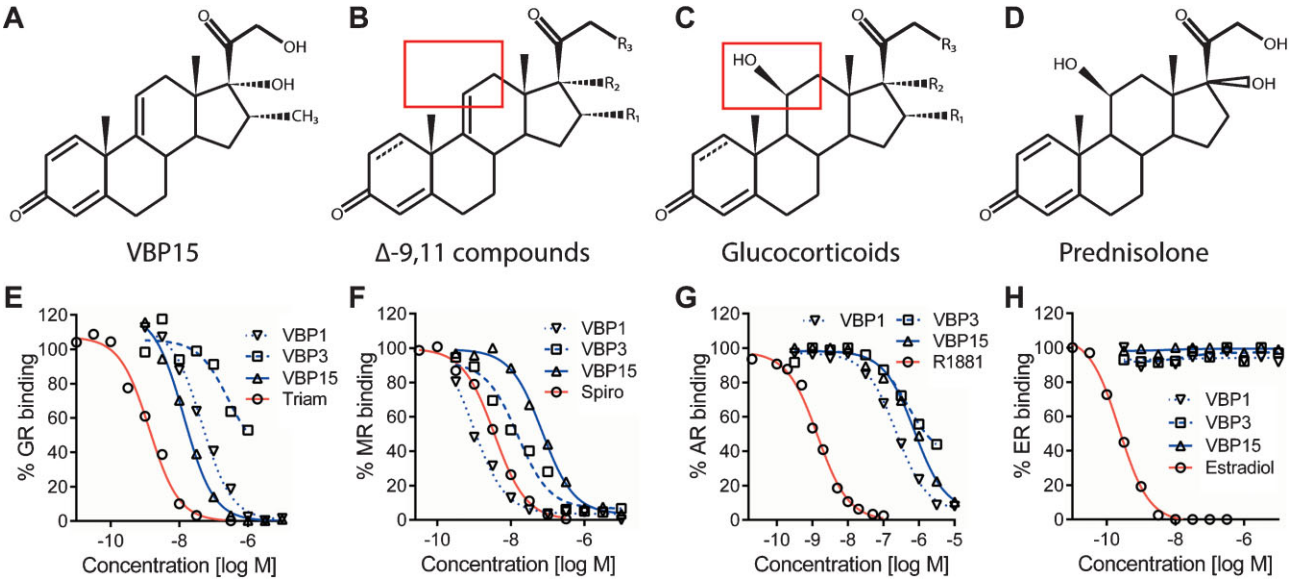


Figure 1. VBP15 structure differentiates it from glucocorticoids and improves GR specificity.

A–D. The chemical structure of VBP15 is provided (A). VBP15 was selected for clinical development by having the optimal profile from a library of Δ-9,11 compounds, whose general structure is provided in (B). Compound diversity for this library was generated by medicinal chemistry probing of the R_1–R_3 groups. These compounds are structurally related to glucocorticoids (C), but contain an essential Δ-9,11 double bond modification to the steroid C-ring, at the location depicted by the red box. This modification produces novel properties and clear differences in sub-activity profiles of these compounds. Prednisolone (D) is the active form of prednisone, a current glucocorticoid standard of care for DMD.

E–H. Receptor specificity was determined through competitive binding assays. Here, radiolabeled high-affinity ligands were incubated with extracted steroid receptors and increasing concentrations of unlabeled competitor (high-affinity control, VBP15, VBP1 or VBP3). Best-fit curves are provided. VBP15 showed increased GR specificity through increased binding to the (E) GR and decreased binding to the (F) MR in comparison to other Δ-9,11 compounds (VBP1 and VBP3). VBP15 also showed low (H) androgen receptor binding and no detectable binding to the (G) oestrogen receptor. (Triam, triamcinolone; Spiro, spironolactone; R1881, methyltrienolone; Estradiol, 17β-estradiol).

inflammatory transcripts *Cox2*, *Irf1* and *Nos2* ($p < 0.005$) at potencies similar to prednisolone (Fig 2B).

Both prednisolone and VBP15 are hydrophobic compounds that are expected to have physicochemical effects on lipid bilayers. We compared the effects of VBP15 and prednisolone on membrane injury and repair in live cells using an established laser injury assay (Sharma et al, 2012). Skeletal muscle cells treated with VBP15 showed reduced impact of the injury and enhanced repair in a dose dependent fashion (Fig 2C and D). Cells treated with prednisolone, however, showed greater impact from injury with elevated dye uptake. In this live single-cell injury model, prednisolone exacerbated, while VBP15 protected, injury to the plasma membrane.

GR mediates VBP15 anti-inflammatory effects without inducing classical steroid transactivation

To investigate whether NF-κB inhibition by VBP15 is mediated by the same pathways as glucocorticoids, we examined the effects of the steroidal receptor antagonist, RU-486, on NF-κB inhibition. Increasing concentrations of RU-486 from 1 nM to 10 μM ablated NF-κB inhibition by VBP15 in a dose dependent manner, similar to results seen with prednisolone and dexamethasone (Fig 3A). This shows that the anti-inflammatory effects of VBP15, prednisolone and dexamethasone are all mediated through shared steroidal pathways.

A sub-activity of pharmacologic glucocorticoids that is largely separable from NF-κB inhibitor activities is the translocation of ligand-GR complexes to the nucleus where they directly mediate transcriptional pathways via glucocorticoid response elements (GRE) (e.g. classical steroid receptor transactivation or hormonal properties). Both positive- and negative-acting GRE-mediated transcriptional regulation has been described, and both forms of hormonal activities are more often associated with glucocorticoid side effects rather than efficacy, with some of these mediated by the pituitary (Diamond et al, 1990; Drouin et al, 1993; Itani et al, 2002; Meijsing et al, 2009; Yoshiuchi et al, 1998). In AtT-20 pituitary cells, we examined genes regulated by positive and negative GREs. *Sgk1*, a key mediator of fibrosis, is activated by a positive GRE. Both prednisone and dexamethasone (0.1 μM) showed a greater than 13-fold induction of *Sgk1* gene transcription, whereas VBP15 showed no such GRE-mediated transcriptional activity at the same concentration (Fig 3B). At 1.0 and 10 μM, VBP15 began to show some evidence of *Sgk1* transcriptional induction, but to a lower degree than traditional glucocorticoids. Adrenocorticotropic hormone (ACTH), the stimulatory hormone in adrenal steroidogenesis, is negatively regulated by a ligand/GR-GRE interaction (Drouin et al, 1993). Treatment with dexamethasone or prednisolone reduced ACTH secretion in AtT-20 cells to approximately 20% of untreated at all concentrations tested

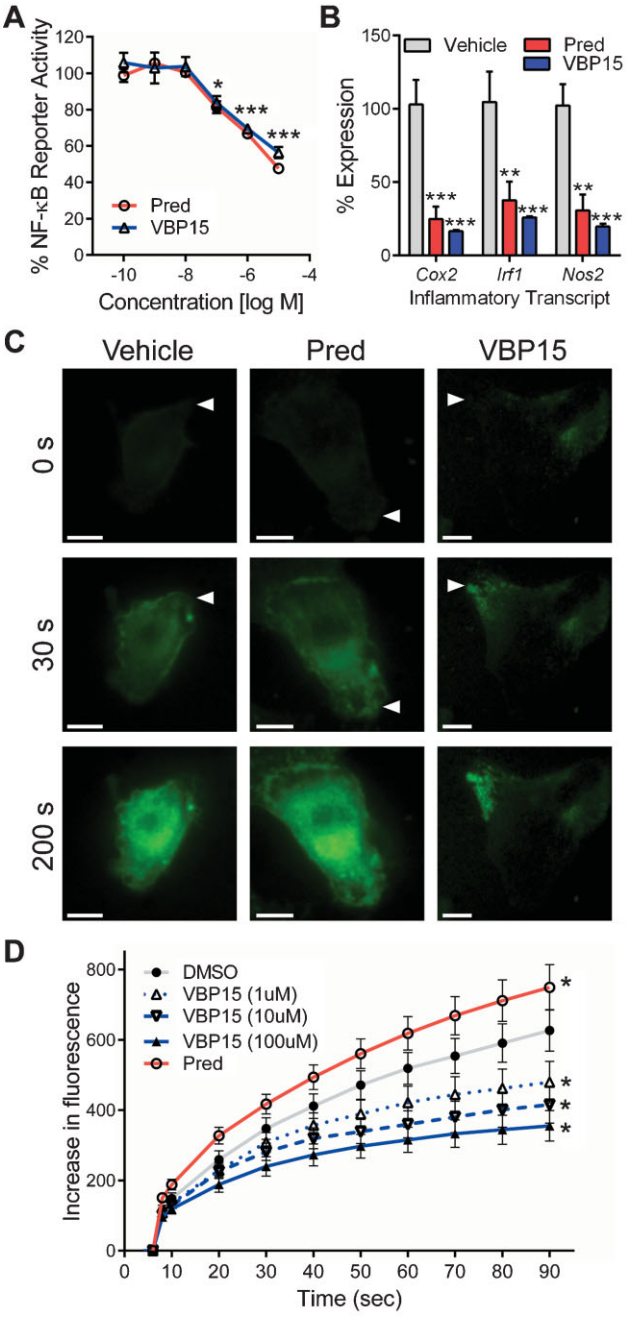


Figure 2. VBP15 inhibits inflammatory signaling and promotes membrane stability.

A. In an NF-κB reporter assay, increasing concentrations of prednisolone or VBP15 were applied to TNFα-induced C2C12 myoblasts stably expressing a luciferase reporter under the control of an NF-κB driven promoter. Significant reporter inhibition was observed at all concentrations over 10 nM.

B. Inhibition was also observed for endogenous NF-κB activated inflammatory transcripts. *Cox2*, *Irf1* and *Nos2* expression was significantly reduced in TNFα-induced H2K myotubes, as determined by real time qPCR. Results are mean ± SEM from representative experiments performed in triplicate (ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

C,D. Laser injury and dye exclusion assays to determine effects of VBP15 and prednisolone on cell membrane integrity. (C) Images of C2C12 myoblasts exposed to the indicated drug 15 min prior to laser wounding, with fluorescent visualization of FM1-43 dye entry into cells (white arrowheads mark sites of injury, scale bars = 5 μm). VBP15 shows protection against laser-induced injury. (D) Quantitation of FM1-43 influx over time (laser injury at time 6 s). VBP15 shows reduced impact of initial injury and enhanced repair, whereas prednisolone shows greater impact from injury with elevated dye uptake. Representative data from one of four experiments are presented as mean ± SEM. (ANOVA, $n \geq 16$ per treatment, $^{*}p < 0.05$; Pred, prednisolone).

(Fig 3C). VBP15 produced more modest, dose-dependent effects on ACTH. qPCR of *Pomc*, the ACTH precursor, confirmed effects were consistent with transcription (data not shown). Thus, VBP15 has greatly reduced effects on both positively and negatively GRE-regulated transcripts in comparison to glucocorticoids, and might be expected to show a more favourable side effect profile.

Several mechanisms have been hypothesized for the inhibition of NF-κB by glucocorticoids and the GR. These include GRE-driven transactivation of genes that inhibit NF-κB, direct protein–protein interactions through which the GR may act as a corepressor when bound to NF-κB, and the activation of alternative receptors such as the MR. To investigate the mechanism by which glucocorticoids, VBP15 and/or the activated GR inhibit NF-κB, we performed further experiments in GR mutant cells. First, we tested whether the lack of GR in GR^{null} mutant fibroblasts affects the ability of drugs to inhibit inflammatory transcripts, which are predominantly controlled by NF-κB. Absence of the GR in this spontaneous mutant line was previously selected for (Housley & Forsthoefel, 1989) and confirmed here through Western blot (Fig 3D). Cells were then treated with prednisolone or VBP15 and inflammatory transcripts were induced with TNFα. Ablation of GR transactivation functions in GR^{null} cells was confirmed through qPCR of *Sgk1* transcript levels (Fig 3E). Examining inflammatory transcripts, we found *Irf1* ($p < 0.0001$), *Tnfα* ($p < 0.05$) and *Il1a* ($p < 0.05$) expression to all be significantly elevated in induced *versus* non-induced cells. In GR positive cells, both VBP15 and prednisolone inhibited the induction of *Irf1* ($p < 0.005$), *Tnfα* ($p < 0.01$) and *Il1a* ($p < 0.05$) to levels that were 30–58% of vehicle (Fig. 3F). In GR^{null} cells, neither drug was able to inhibit the induction of any of these transcripts. This data confirms that ligand-activated GR is essential for the inhibition of predominantly NF-κB driven inflammatory transcripts by both prednisolone and VBP15.

Next, primary splenocytes were harvested from control and GR^{dim} mutant mice. These mice contain a mutation in the DNA binding domain of the GR (Dahlman-Wright et al, 1991; Reichardt et al, 1998). This mutation prevents the GR from binding to DNA and activating dimer-driven GRE gene transcription, but maintains GR ligand-binding and protein–protein interactions. Here, primary splenocytes were treated with drug and induced with TNFα, then assayed by qPCR. First, we examined the induction of NF-κB inhibitor alpha (*Nfkbia*, or *IκBα*), a GRE-activated gene that also encodes an endogenous

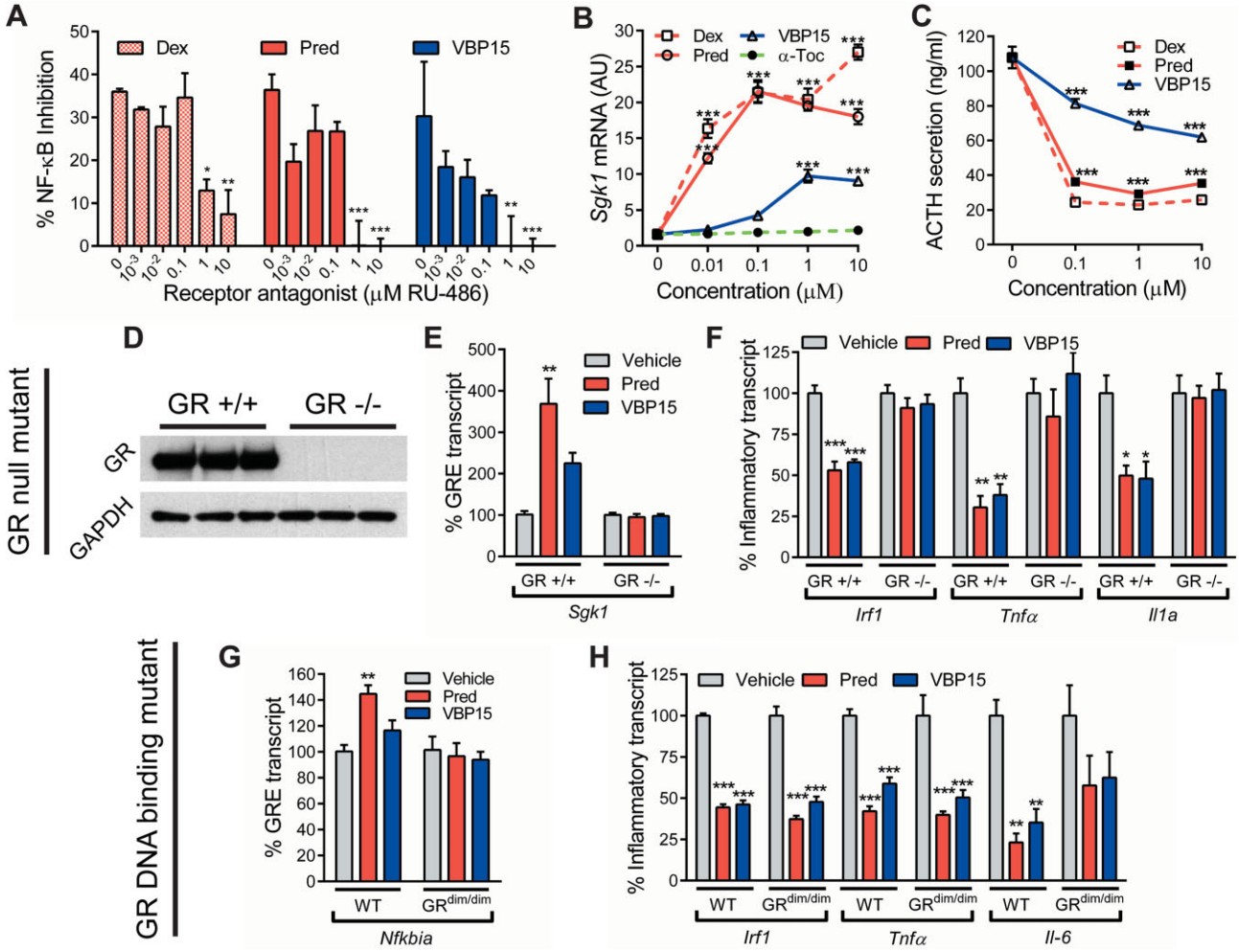


Figure 3. GR is required for anti-inflammatory activities of VBP15 and prednisolone, but VBP15 shows loss of GRE-mediated sub-activities associated with side effects.

A. Application of a steroidal receptor antagonist (RU-486) ablated the NF-κB inhibitory activity of both prednisolone and VBP15, indicating that both drugs share steroidal anti-inflammatory pathways.

B,C. Assessment of GRE-mediated transcriptional (hormonal) activities differentiates prednisolone and VBP15. (B) *Sgk1* gene expression is controlled by a positive GRE and showed reduced activation by VBP15 in comparison to glucocorticoids in AtT-20 pituitary cells, as measured by qRT-PCR. α-Tocopherol was included as a negative control compound that lacks any GRE activity. (C) ACTH expression is controlled by a negative GRE, and is considered a component of adrenal suppression (negative side effect of pharmacological glucocorticoids). VBP15 showed reduced effects on this side effect pathway via ELISA of treated AtT-20 cell media.

D. Western blot of GR^{null} fibroblasts and the control GR positive L929 line they were derived from, illustrating the absence of detectable GR protein in the GR^{null} cells. GAPDH was included as a loading control.

E,F. GR^{null} and GR positive fibroblasts were treated with drug, then induced with TNFα and transcript levels assayed by real time qPCR. (E) *Sgk1* levels illustrate an absence of induction in GR^{null} cells, confirming *Sgk1* dependence on the GR and the absence of GR function in this cell line. (F) Inhibition of the endogenous NF-κB activated inflammatory transcripts *Irf1*, *Tnfα* and *Il1a* was observed in GR positive cells but not in GR^{null} cells, indicating the GR is essential for this inhibition.

G,H. Spleens were harvested from $GR^{dim/dim}$ and wild type control mice. Splenocyte suspensions were treated with drug, then induced with TNFα and transcripts assayed by qPCR. (G) *Nfkbia* levels illustrate an absence of GRE induction by the mutant GR, as well as a lack of induction of NF-κB inhibiting gene products, in $GR^{dim/dim}$ splenocytes. (H) Inhibition of *Irf1*, *Tnfα* and *Il6* inflammatory transcripts was observed in both wild type and $GR^{dim/dim}$ mutant splenocytes. This indicates GR^{dim} isoforms, which maintain protein-protein interactions but lose receptor-DNA interactions, still maintain inhibition of endogenous NF-κB activated inflammatory transcripts. (Pred, prednisolone; Dex, dexamethasone; α-Toc, vitamin E; ANOVA, $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

inhibitor of NF-κB. In wild type control splenocytes, *Nfkbia* expression was significantly increased by prednisolone (increase of $45 \pm 13\%$, $p < 0.005$) but not by VBP15 (increase of $16 \pm 16\%$) in comparison to vehicle. No induction was present with either drug in GR^{dim} splenocytes, demonstrating both the absence of GR dimer-driven gene expression in GR dim cells and a lack of induction of NF-κB inhibitory genes. Examining inflammatory transcripts, we found *Irf1* ($p < 0.0001$), *Tnfα* ($p < 0.0001$) and *Il6*

($p < 0.05$) were significantly elevated within induced *versus* non-induced primary splenocytes. Consistent with GR positive fibroblasts and H2K myotubes, treatment of wild type splenocytes with both VBP15 and prednisolone successfully inhibited the induction of all three inflammatory transcripts ($p < 0.001$) to levels that were roughly half those of vehicle. In contrast to GR^{null} genotype and GRE transcript experiments, we found that inhibition of all three inflammatory transcripts was maintained in the GR^{dim} mutant cells. Together, these experiments show that both prednisolone and VBP15 activate the GR to efficiently inhibit inflammatory transcription programs through protein–protein interactions, independent of DNA binding or transactivation of inhibitory genes.

VBP15 improves dystrophic phenotypes in mice treated before the onset of early necrosis

The *mdx* mouse model of DMD shows staged histopathology, with little evidence of dystrophy from 0 to 3 weeks of age, then wide-spread necrosis from 3 to 6 weeks, followed by successful regeneration and a milder, more stable histological picture. We tested efficacy of VBP15 in the *mdx* model with treatment beginning prior to the 3 weeks onset of widespread pathology (prophylactic strategy). We carried out a blinded pre-clinical trial of pre-symptomatic mice with VBP15 (5, 15 or 30 mg/kg), prednisolone (5 mg/kg), or vehicle beginning at postnatal day 15 (PND15), following guidelines for robust pre-clinical trials and international SOPs (Landis et al, 2012; Nagaraju & Willmann, 2009; Spurney et al, 2009). These doses were chosen on the basis of favourable bioavailability, ADME and metabolite profiles (Reeves et al, 2013), as well as early safety studies in wild type mice by independent groups, which suggest the 28 day no-observable adverse effect level (NOAEL) of daily oral VBP15 in mice is at least 100 mg/kg. This dose range was chosen to better define the therapeutic window within this mouse disease model. The prednisolone dose was chosen based on our extensive pre-clinical experience with this drug in the *mdx* mouse model.

Both VBP15 and prednisolone increased *mdx* forelimb and hindlimb normalized grip strength in comparison to vehicle (Fig 4A and B). Significant increases in VBP15 groups followed a dose-dependent pattern from 14% at 5 mg/kg ($p < 0.05$) to 20% at 30 mg/kg ($p < 0.0005$). For maximal force exerted, we again saw a dose dependent increase in forelimb strength upon VBP15 treatment, while prednisolone actually showed a reduction in maximal forelimb strength (Fig 4C). The discrepancy between prednisolone's effects on maximal and normalized force measures was due to the marked retardation of mouse growth induced by prednisolone, but not by VBP15 (see below). This indicates VBP15 increases functional mouse limb strength.

Evaluating muscle strength of isolated muscles *ex vivo*, extensor digitorum longus (EDL) muscles showed a reduction in specific force for *mdx* compared to WT (Fig 4D). While prednisolone showed no increase, specific force increased with VBP15 at both 15 and 30 mg/kg by an average of 12%. Following lengthening contractions, smaller drops in force for *mdx* EDLs after 10 contractions were observed for mice treated with prednisolone (7%) and VBP15 (11% at 15 mg/kg, $p < 0.05$), in comparison to vehicle (Supporting Information Fig 1A). These data suggest functional benefits to isolated dystrophic muscles.

Optical imaging of live animals was used to monitor muscle inflammation. ProSense 680, a substrate cleaved by cathepsin proteases upregulated in DMD (Kar & Pearson, 1978; Takeda et al, 1992), was injected as previously reported (Baudy et al, 2011). Cathepsin activity was elevated in *mdx* mice (Fig 4E, Supporting Information Fig 1B and C). VBP15 and prednisolone decreased cathepsin activity towards WT levels. Decreases in VBP15 groups followed a dose-dependent pattern, from a 22% decrease in comparison to vehicle at 5 mg/kg to a 41% decrease at 30 mg/kg in hindlimbs. This suggests VBP15 reduces muscle inflammatory disease *in vivo*.

In histopathology studies, quantitative H&E analysis of *mdx* diaphragms revealed a clear inflammatory phenotype, with 16-fold higher inflammatory cell counts compared to WT (Fig 4F). Mice treated with VBP15 at 15 and 30 mg/kg displayed 38 and 30% reductions in inflammatory foci compared to vehicle. VBP15 also reduced calcified fibres (Supporting Information Fig 1D). These data are evidence that VBP15 reduces inflammation and improves inflammatory muscle pathology.

VBP15 improves dystrophic phenotypes in adult *mdx* mice treated after symptom onset

In a separate trial, exercised adult *mdx* mice were treated for 4 months. In agreement with the pre-symptomatic trial above, ProSense680 live animal imaging exhibited a 20% and 13% decrease in muscle inflammation upon VBP15 treatment at 15 and 45 mg/kg (Fig 4G). Isolated EDLs showed a 16% increase in specific force upon treatment with VBP15 at 15 mg/kg (Fig 4H). H&E histology revealed VBP15 significantly decreased diaphragm inflammation (Fig 4I). These data reinforce VBP15 efficacy and indicate both pre-symptomatic and post-onset treatment regimens can benefit disease.

VBP15 does not display immunotoxicity seen with prednisolone

Pharmacologic glucocorticoids show immunosuppressive and immunotoxic properties that limit therapeutic windows and long-term prescription. We benchmarked VBP15 against prednisolone to determine if similar sub-activities were seen. Untreated *mdx* mice showed enlarged spleens and increased numbers of peripheral blood leucocytes (PBLs) compared to WT mice (Supporting Information Fig 2A and B). VBP15 treatment reduced spleen mass and PBL counts in a dose-dependent manner to levels resembling WT. Prednisolone reduced these measures below WT, suggesting immunosuppressive and/or immunotoxic properties. Further, prednisolone significantly decreased viable splenocytes per gram of tissue ($p < 0.005$), while this was not observed for any VBP15 dose (Fig 5A).

We next examined effects of VBP15 on B and T lymphocytes isolated from *mdx* spleens at the trial conclusion. Both B lymphocytes and $CD4^+$ T lymphocytes were depleted by prednisolone but not VBP15, as measured by percent $B220^+$ and $CD4^+$ positive splenocytes, respectively (Fig 5B and C). $CD4^+$ T cell activation was assayed by stimulation of splenocytes with concanavalin A (ConA). Prednisolone treatment significantly

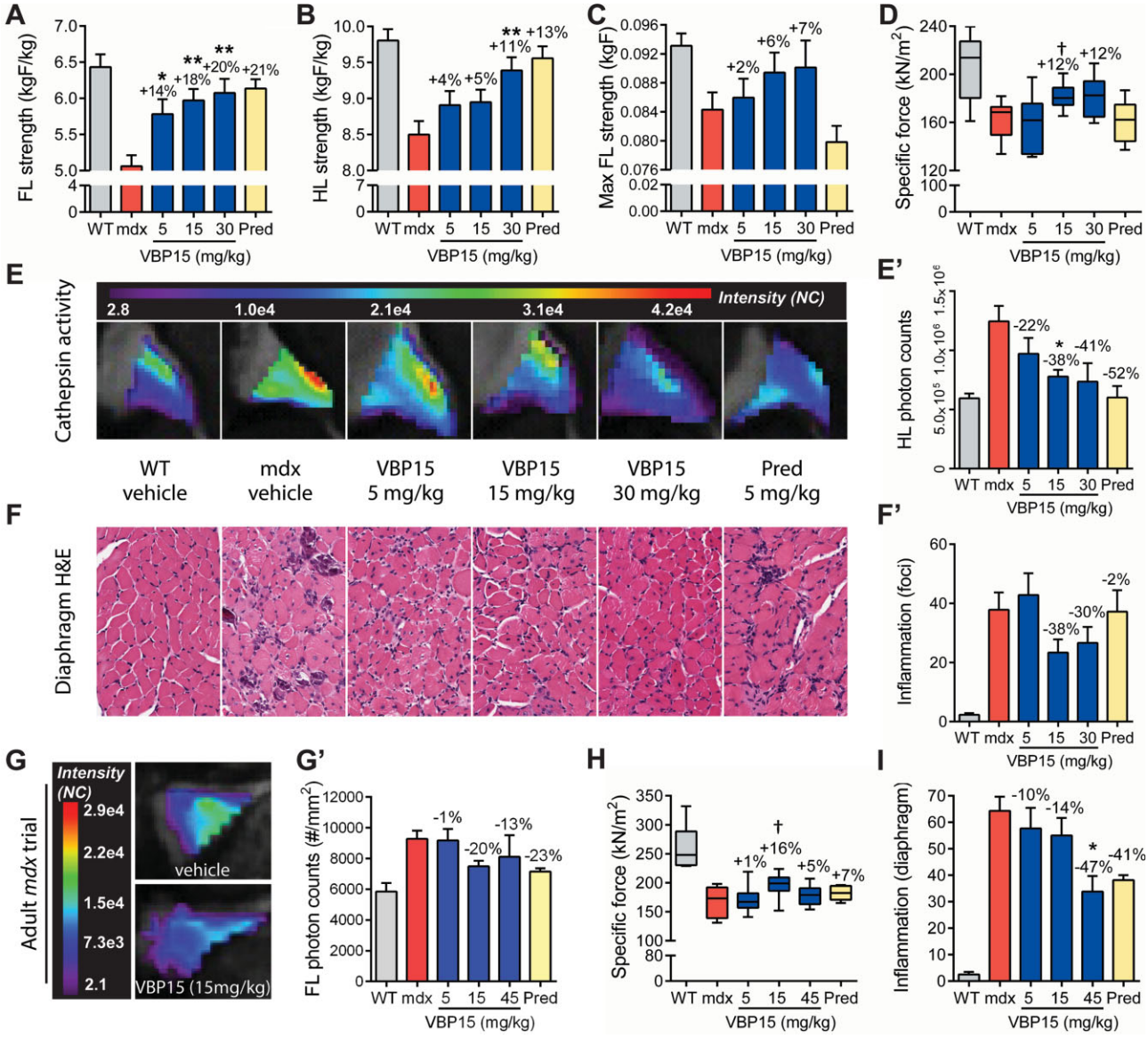


Figure 4. VBP15 improves dystrophic phenotypes of *mdx* mice in two pre-clinical trials (pre-symptomatic and post-onset treatment regimens).

A–F. Prophylactic treatment of *mdx* mice beginning at 2 weeks of age showed dose-dependent improvement of clinical and histological endpoints. Mouse limb strength increased upon VBP15 treatment as measured by grip strength of 6 week old mice for both (A) forelimb and (B) hindlimb ($n \geq 12$ mice/group). (C) Maximal force exerted by mouse forelimbs increased with VBP15 treatment but decreased with prednisolone treatment, due to prednisolone effects on mouse size (presented later). (D) Specific force of isolated EDL muscle increased with VBP15 treatment ($n = 10$ mice/group). (E) Live-animal imaging of cathepsin protease activity (ProSense680) shows reduced inflammation and necrosis of the hindlimbs in VBP15-treated *mdx* mice (E images; E′ quantitation of fluorescence; $n \geq 6$ mice/group). (F) Histology of diaphragm muscle shows a decrease in inflammatory foci from VBP15 treatment at 15 and 30 mg/kg (F representative images, F′ quantitation; $n = 6$ mice/group).

G–I. A second pre-clinical trial was performed in exercised adult *mdx* mice to assay post-onset efficacy. (G) Live-animal imaging of inflammation (ProSense680) showed a significant decrease with VBP15 treatment (G representative images, (G′) quantitation; $n \geq 6$ mice/group). (H) Specific force of isolated EDL muscle was measured *ex vivo* at trial conclusion with 15 mg/kg VBP15 showing an increase consistent with the neonate trial ($n \geq 7$ mice/group). (I) Histology of adult diaphragm showed a significant reduction in inflammatory foci upon 45 mg/kg VBP15 treatment ($n = 6$ mice/group). Values are mean ± SEM. For treatments, the mean percentage of increase or decrease of *mdx* vehicle values towards WT is provided. (Pred, prednisolone; FL, forelimb; HL, hindlimb; data exceeding 2 SD's was removed from specific force values as an outlier but included in all statistical analyses; one-tailed *t*-test of single dose *versus* vehicle *mdx* $^{\dagger}p < 0.05$; ANOVA of dose-dependence groups *versus* vehicle *mdx* $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

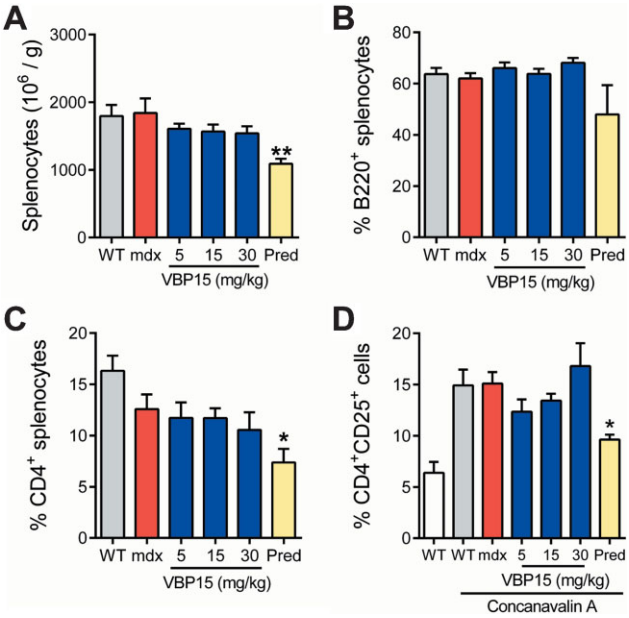


Figure 5. VBP15 does not show immunosuppressive activities shown by prednisolone.

A. Prednisolone significantly reduced the number of viable splenocytes per gram of spleen tissue, whereas VBP15 did not at any dose.

B. The percentage of B lymphocytes, as measured by FACS analysis of B220 positive cells, was reduced in spleens from prednisolone treated *mdx* mice, while VBP15 showed no decrease in B cells.

C. Spleen $CD4^+$ T cell numbers were significantly decreased in prednisolone treated *mdx* spleens, but not by VBP15 treatment.

D. Activation of *mdx* splenocyte T cells by concavalin A was impaired by prednisolone, but not impaired by VBP15 treatment. Values are mean ± SEM. (Pred, prednisolone; (A) $n \geq 12$, (B–D) $n = 3$–5; $^*p \leq 0.05$, $^{**}p < 0.005$).

reduced activated $CD4^+CD25^+$ cells ($p = 0.01$), while VBP15 did not. Taken together, these findings suggest VBP15 modulates inflamed *mdx* immune systems towards a WT state, while prednisolone treatment leads towards an immunocompromised state.

VBP15 shows a superior side effect profile compared to pharmacological glucocorticoids

Stunted growth is a significant side effect of chronic prednisone use in children (Avioli, 1993; Wolthers & Pedersen, 1990). In our pre-symptomatic *mdx* study, prednisolone treatment significantly stunted the growth of young mice (Fig 6A). After 5 weeks of treatment, *mdx* mice receiving prednisolone were significantly shorter (8.6 ± 0.4 cm) than vehicle (9.1 ± 0.3 cm, $p < 0.001$). No significant reduction in body length was observed for any VBP15 dose.

Chronic treatment with glucocorticoids negatively affects bone growth and development, and can cause osteoporosis (Bircan et al, 1997; Manolagas & Weinstein, 1999). Tibia length was measured to determine if VBP15 inhibited bone growth (Fig 6B). Vehicle *mdx* mice had tibia lengths of 15.9 ± 0.3 mm, while prednisolone significantly decreased this to 14.8 ± 0.5 mm ($p < 0.005$). VBP15, however, did not affect tibia length at any concentration. MicroCT was performed on femurs to examine bone density and structure (Fig 6C). Comparison of vehicle, prednisolone and the highest VBP15 dose showed prednisolone to significantly reduce trabecular thickness ($p < 0.005$) compared to vehicle, while VBP15 did not. Prednisolone thus demonstrated side effects to bone not observed with VBP15 treatment.

We have previously reported deleterious effects of prednisone on increased fibrosis in *mdx* hearts (Sali et al, 2012). In both pre-clinical trials (pre-symptomatic and adult), we examined cardiac and skeletal muscle for measures of fibrosis. In the pre-symptomatic trial (Fig 6D–F), prednisolone caused a significant elevation of heart mass ratios over vehicle (5.9 ± 0.6 *vs.* 5.4 ± 0.4, $p < 0.05$), indicative of cardiac hypertrophy. No increase was present in VBP15 groups. Histologically, clear fibrosis was evident in 50% of young (8 weeks) prednisolone-treated hearts compared to 0% of all other groups. Histological analyses of skeletal muscle (gastrocnemius) also showed increased fibrosis in prednisolone-treated mice (8.1 ± 2.2%, $p < 0.05$) compared to vehicle-treated (4.2 ± 1.8%), VBP15-treated (3.5 ± 1.2% at 30 mg/kg), and WT (2.0 ± 0.5%) mice (Supporting Information Fig 2C–E). In the adult trial, cardiac findings were consistent with the pre-symptomatic trial (Fig 6G and H). Here as well, prednisolone treatment increased fibrosis and mass ratios of *mdx* hearts, while VBP15 did not.

DISCUSSION

Development of mechanisms to improve muscular dystrophy in the absence of detrimental hormonal effects will substantially improve DMD patient medical care, could provide a therapy for dystrophies with no current treatment, and could improve care of diverse chronic inflammatory disorders. Here, we describe the development, mechanisms and effects of a novel drug that dissects and optimizes several sub-activities of classic glucocorticoids (Fig 7), demonstrating it is possible to treat muscular dystrophy in the absence of growth, hormonal and immunosuppressive side effects. For one sub-activity, we show VBP15 has protective physicochemical effects on the plasma membrane, protecting cells from injury and promoting membrane repair. This sub-activity is likely to be particularly important in DMD where disease pathogenesis is clearly linked to membrane instability and myofibre injury. For another, we show that a key anti-inflammatory activity, inhibition of TNFα-induced NF-κB, is retained by VBP15. We further show that this mechanism occurs through protein–protein interactions of the VBP15 ligand-activated GR, independently of DNA binding, GRE activation, or upregulation of inhibitory transcripts. We have previously shown that NF-κB activation is among the earliest histological features of DMD neonates (Chen et al, 2005; Porter et al, 2002, 2003), years before symptoms appear. This, coupled with the results of our blinded *mdx* pre-clinical data here, suggests that very early treatment of DMD patients with VBP15 may prevent or delay the onset of some clinical symptoms. Finally, the well-documented and extensive side

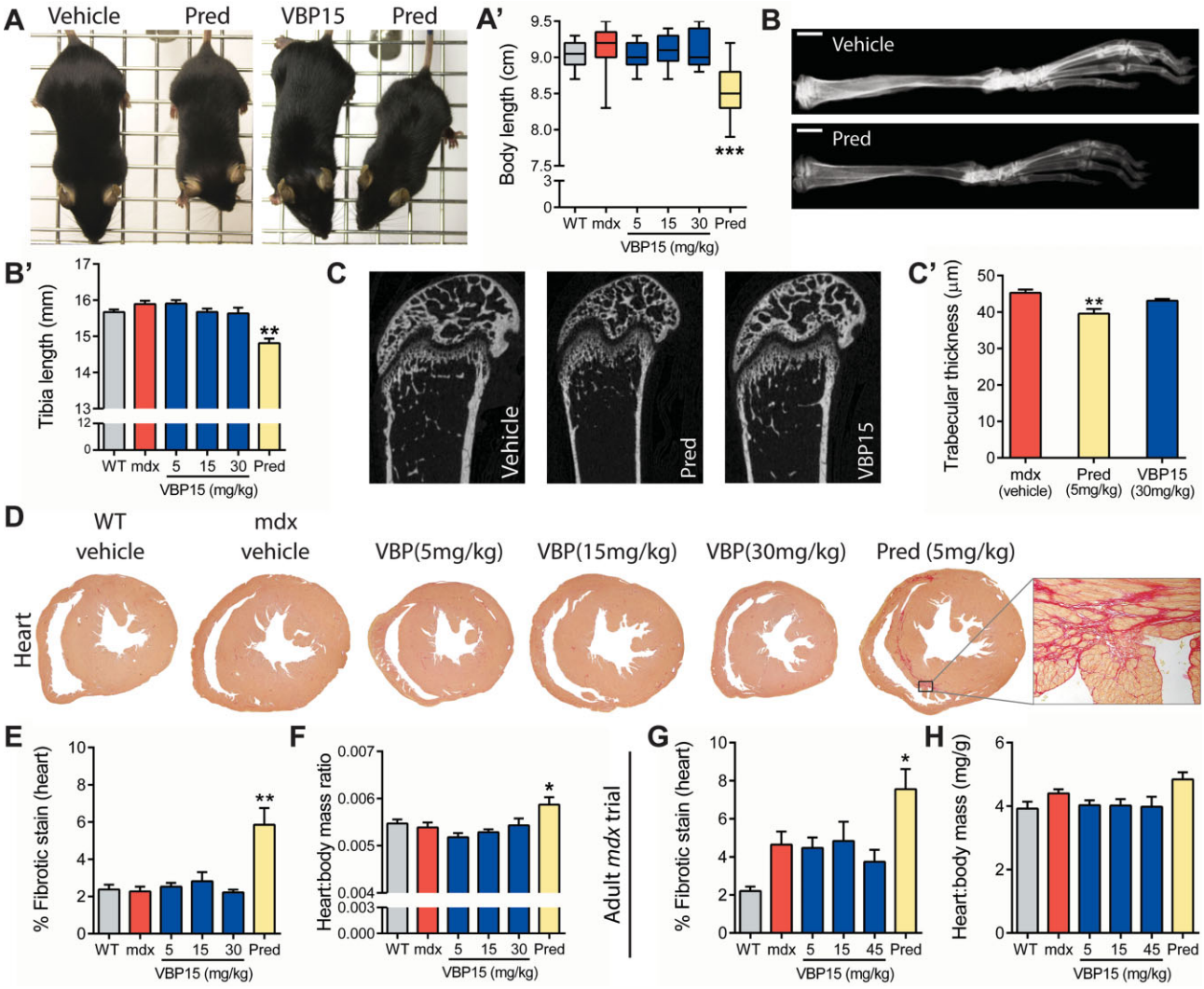


Figure 6. VBP15 lacks the side effects of current glucocorticoid regimens *in vivo*.

A. Prednisolone treatment stunted the growth of developing mice in comparison to both vehicle and VBP15 groups. Representative photographs (A) and quantitation of body length (A′) are provided.

B. Bone lengths were reduced upon prednisolone treatment. X-rays (B) of mouse tibias illustrate size differences (scale bars = 2 mm). Quantitation shows a significant decrease in tibia length (B′).

C. MicroCT imaging analysis of femur revealed a significant decrease in trabecular thickness (C′) for prednisolone treated mice.

D–F. Increases in cardiac fibrosis and heart mass were detected in prednisolone treated mice, suggestive of cardiac damage as a side effect lacking for VBP15. Sirius red staining of cardiac muscle shows increased fibrosis in prednisolone-treated mice, but not VBP15 mice. Representative images (D) and digital quantitation of fibrosis (E) are provided. To the right of the image panel is a higher magnification image from the area outlined in box. (F) Heart mass ratios were increased by prednisolone but not by VBP15.

G,H. In adult *mdx* mice as well, increases in cardiac fibrosis (G) and heart mass (G) were observed with prednisolone treatment but not VBP15 treatment. In adult *mdx* vehicle mice, an expected disease- and age-related increase in fibrosis over WT is seen. Values are mean ± SEM. ($n \geq 12$ per group for (A,B,E,F); $n \geq 5$ for (C,G,H); $^{*}p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$).

effect profiles of glucocorticoids, inclusive of immunotoxicity, growth stunting and effects on pituitary function, were not seen with VBP15 at doses up to nine times prednisolone dosing. These properties provide us with a new mechanistic profile with which to approach both patient therapy and scientific questions regarding inflammation, signaling and disease mechanisms.

Steroidal compounds are multi-mechanistic by nature and display physicochemical effects on the plasma membrane (Rhen et al, 2003; Shivaji & Jagannadham, 1992). We find VBP15 and prednisolone differ in their effects on membranes, with VBP15 treatment protecting live cells from laser-induced injury. Membrane-stabilization is a property that is analogous to poloxamer 188, Mitsugumin 53 or cromolyn sodium, which

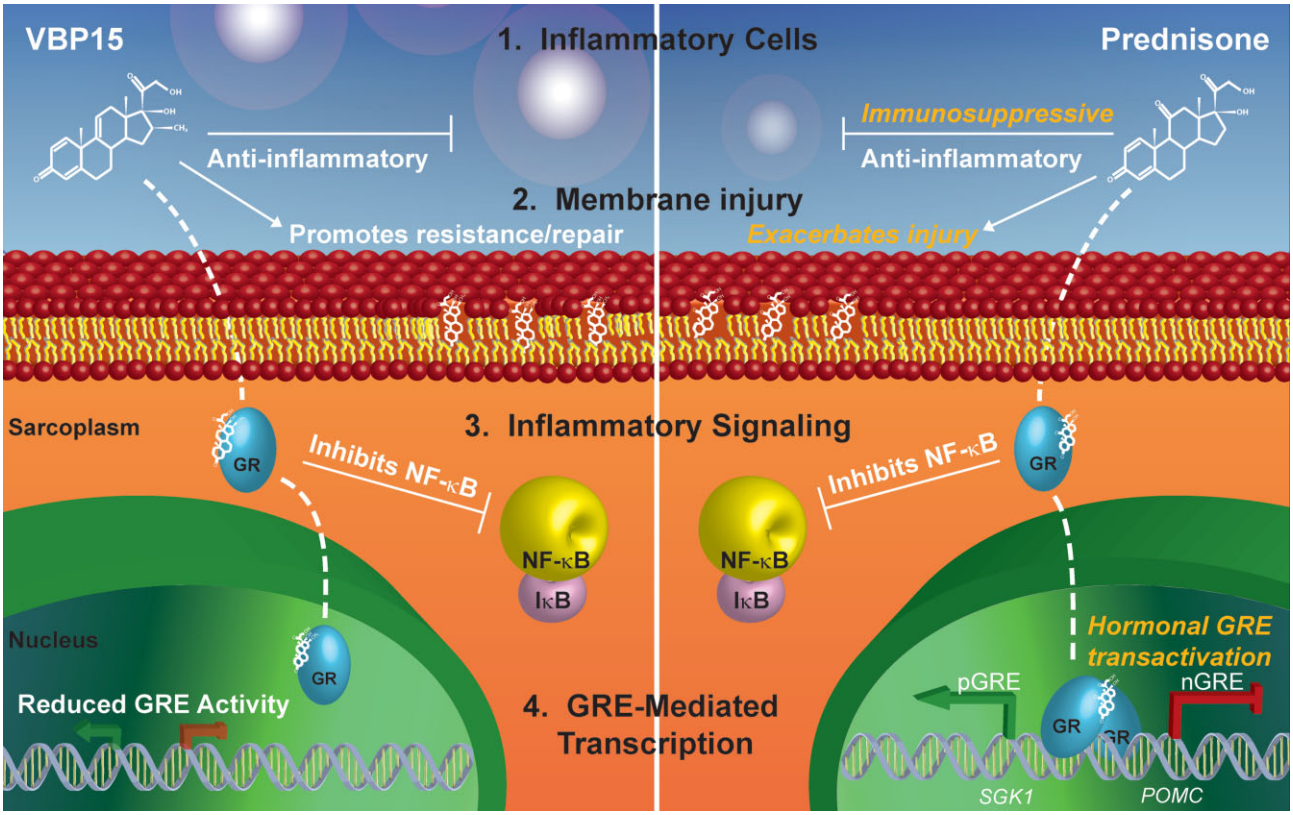


Figure 7. Working model of VBP15 and prednisone drug mechanism sub-activity profiles. Steroidal compounds such as glucocorticoids (prednisone) and Δ-9,11 compounds (VBP15) are multi-potent drugs. Through dissecting the sub-activities of these compounds, we find that: (1) VBP15 reduces inflammation but does not show the immunosuppressive impairment of lymphocyte viability and function observed for prednisone. (2) Within an environment of plasma membrane disruption, VBP15 helps to promote resistance to and repair of injuries, while prednisone can exacerbate membrane injury. (3) Inside cells, both compounds bind to and activate the GR to potently inhibit inflammatory NF-κB signaling through protein–protein interactions. (4) Though they both bind to the GR, prednisone causes strong induction of hormonal GRE controlled promoter elements, while VBP15 eliminates or greatly reduces these effects.

operate through varying mechanisms and show beneficial effects on dystrophin deficient dog and mouse muscle *in vivo* (Marques et al, 2008; Townsend et al, 2010; Weisleder et al, 2012). Membrane-stabilizing effects of VBP15, but not prednisolone, are consistent with increases in specific force observed for VBP15 but not for prednisolone. VBP15 effects on membrane stability could be explained by altered compression of phospholipid head groups within the membrane, altered ion balances (Howard et al, 2011), altered membrane or vesicular fusion (Shivaji & Jagannadham, 1992), or altered oxidative stress at the plasma membrane (Howard et al, 2011; Kavanagh & Kam, 2001; Marques et al, 2008; Saija et al, 2001). With membrane integrity and repair becoming of increasing importance in muscle (Bansal et al, 2003; Jaiswal et al, 2007), cardiovascular (Chase et al, 2009), neurodegenerative (Bazan et al, 2005) and airway (Gajic et al, 2003) disorders, physicochemical properties of VBP15 will be an intriguing area of investigation moving forward.

Chronic treatment with glucocorticoids (prednisone, deflazacort) is the current standard of care for DMD, yet glucocorticoids are well-known to induce muscle atrophy pathways via *FOXO1*, stunt the growth of paediatric patients, and can suppress the immune system which plays an important role in myofibre repair cycles. Thus, clinical improvements in DMD patients treated with glucocorticoids may be the sum balance of beneficial anti-inflammatory effects and deleterious pathways. Both *in vitro* and *in vivo* data presented here are consistent with this model. In *mdx* mice, we find the net balance of prednisolone treatment increases normalized strength, however at the same time it stunts the growth of mice resulting in lower maximal strength, is immunosuppressive, and increases the presence of muscle damage. We, as well as others in recent reports (Bauer et al, 2009), also find that prednisolone increases cardiac fibrosis in *mdx* mice. Comparable examination of cardiac fibrosis in glucocorticoid treated DMD patients has not been examined directly in the literature, however recent anecdotal cardiac MRI reports show substantial fibrosis, suggesting this may be an intriguing area of investigation moving forward, with a possibility to develop more "heart healthy" treatments. VBP15 however does not stunt the growth of mice, shows no evidence of splenocyte immunotoxicity, and does not increase muscle fibrosis in skeletal or cardiac muscle. In

the absence of these side effects, VBP15 increases strength, increases absolute and specific force measures and decreases muscle inflammation. A comparison of VBP15 and prednisolone mechanistic profiles in the context of these results has several implications. One, stunted growth appears to be a side effect of glucocorticoid treatment in DMD as opposed to a mechanism of efficacy, as has been logically proposed in the past (Grounds & Shavlakadze, 2011) by limiting body size to reduce muscle workload. Here, we see dose-dependent increases in *mdx* strength in the absence of stunted growth, suggesting the potential to increase patient strength without overt effects to growth and development. Two, immunotoxicity is a potential side effect of glucocorticoid treatment – not the primary cause of efficacy. This is supported by the failure of general immunosuppression to increase DMD patient strength (Griggs et al, 1993; Kissel et al, 1993). Three, NF-κB inhibition is shared by both drugs, supporting our hypothesis that this is a shared pathway of efficacy. In further support of this, peptides or antibodies targeting NF-κB pathways benefit *mdx* phenotypes (Grounds & Torrisi, 2004; Peterson et al, 2011), while in contrast, constitutive activation of NF-κB causes severe muscle wasting (Cai et al, 2004; Mourkioti et al, 2006). Finally, GRE transactivation appears to be disposable to efficacy in DMD. As GRE-regulated genes have been implicated in a number of glucocorticoid side effects, this is particularly exciting because it provides a clear avenue by which to reduce harsh side effect profiles currently limiting the use of these widely applicable drugs. Indeed, along with a reduction in GRE activity for VBP15, we also see an absence of glucocorticoid side effects in *mdx* mice. Importantly, efficacy is maintained in the absence of immunosuppression and overt hormonal effects to growth and development, providing insight into mechanisms of glucocorticoids in muscular dystrophy and demonstrating a successful separation of pathways into dystrophy efficacy and side effects.

Exon skipping represents another promising line of therapeutic development for DMD (Hoffman et al, 2011). Short of gene replacement, this may offer the greatest potential to alleviate DMD because it aims to restore expression of disease-causing dystrophin deficiencies. However, isoforms induced by exon skipping, as well as the mini-gene dystrophin constructs envisioned in gene therapy, are also expressed in Becker muscular dystrophy (alleles of dystrophinopathy leading to milder disease). In other words, both exon skipping and gene therapy are expected to mitigate but not cure disease. We and others find exon skipping partially restores specific force deficits in *mdx* muscle, with *ex vivo* force contractions typically showing approximately 20% increases over *mdx* controls (Aoki et al, 2010; Dumonceaux et al, 2010). This likely represents an upper limit to therapeutic strength increases, short of gene replacement. We find VBP15 treatment increases EDL specific force as well, with 15 mg/kg producing 12 and 16% increases in the two trials presented here. Currently, patients with Becker or other milder muscle dystrophies are not routinely administered prednisone (Johnsen, 2001) due to the unclear net balance of detrimental *versus* beneficial effects it would provide. Evidence here suggests VBP15 could provide a novel therapy for Becker's and other milder dystrophies, or serve as a valuable combination therapy used with exon skipping to provide efficacy through independent mechanisms.

Currently, glucocorticoid regimens for DMD delay treatment to avoid serious detriment, and many patients eventually discontinue treatment as a result of side effects. A drug lacking such harsh effects has the potential to change physicians' treatment approaches since it would be more amenable to a chronic treatment regimen, and would enable treatment during pre-symptomatic or late stages when many patients are not taking prednisone. This rationale prompted us to change our approach to *mdx* preclinical trial design for VBP15, and indeed we saw clear efficacy with an ablation of side effects to *mdx* growth, bone and muscle. Intriguingly, by enabling treatment of DMD at pre-symptomatic ages, a strong rationale for neonatal screening could be built to move forward towards a preventative medicine approach to treatment, thereby improving the way we diagnose and treat DMD patients.

International consensus has established the *mdx* mouse as the model of choice for preclinical and proof-of-concept studies because they represent the exact monogenic biochemical defect present in DMD (Nagaraju & Willmann, 2009; Willmann et al, 2009, 2012). However, *mdx* mice present a milder disease than DMD, with peak severity from approximately 3–8 weeks of age after which they recover substantially until advanced ages. This prompts various strategies to exacerbate the *mdx* phenotype. One strategy is to introduce additional mutations which exacerbate disease onto the *mdx* background, examples of which include the *mdx:utrophin*$^{-/-}$ (Deconinck et al, 1997; Grady et al, 1997b), *mdx:adbn*$^{-/-}$ (Grady et al, 1999), *mdx:α7 integrin*$^{-/-}$ (Guo et al, 2006), *mdx:PV*$^{-/-}$ (Raymackers et al, 2003) and *mdx:MyoD*$^{-/-}$ (Megeney et al, 1999) double knockout models. These provide advantages through increased disease severity and a differing array of symptoms, which allow for more efficient trials utilizing smaller sample sizes without the added need of forced exercise protocols. Several groups have thus utilized *mdx:utrophin*$^{-/-}$ double transgenic mice to successfully detect therapeutic efficacy (Delfin et al, 2011; Gehrig et al, 2012; Goyenvalle et al, 2010; Wakefield et al, 2000). It is possible for a second mutation to introduce underlying biochemical or biological differences however, for example *utrophin*$^{-/-}$ single transgenic mice have an increased susceptibility to seizures (Knuesel et al, 2002), along with altered neuromuscular junction folding and altered acetyl choline receptor density (Grady et al, 1997a), which could feasibly affect neuromuscular disease outside of a direct consequence of dystrophin deficiency (Willmann et al, 2009). Here, we chose to use larger sample sizes of monogenic *mdx* mice to ensure that the efficacy parameters we measured were from phenotypes directly resulting from dystrophin deficiency. To optimize our trial designs, we adopted two strategies to measure *mdx* phenotypes at points of increased severity, in one trial by assaying young mice during natural peaks in disease severity, and in the other by using forced exercise protocols in adult mice to exacerbate disease. Through both strategies, we consistently detect significant *mdx* phenotypes and VBP15 efficacy through an improvement of *mdx* phenotypes towards wild type.

Extension of VBP15 to other clinical disorders of membrane instability and chronic inflammation will require further studies and clinical development. Studies of VBP15 in animal models of arthritis, asthma, multiple sclerosis and inflammatory bowel diseases are currently underway. Through collaboration with the Muscular Dystrophy Association Venture Philanthropy, and the National Institutes of Health Therapeutics for Rare and Neglected Disease (TRND) program, VBP15 is being actively developed for DMD as the initial indication. Participation in these programs has provided repeatability, efficacy and safety through independent trials both *in vitro* and *in vivo*. VBP15 shows favourable pharmacokinetic, ADME and metabolite profiles (Reeves et al, 2013). Early safety studies by independent groups suggest a single dose tolerance of at least 500 mg/kg in mice, and a 28 day NOAEL of at least 100 mg/kg in mice, which is more than twice the highest doses (30 and 45 mg/kg) used here to show both efficacy and a clear reduction in side effects in comparison to prednisolone.

Our results demonstrate the successful separation of pathways providing efficacy from side effects in muscular dystrophy. The translation of these into model mice by treatment with a novel, orally available drug, indicates that strength and pathology phenotypes can be improved by treatment without overt hormonal, growth or immunosuppressive effects. VBP15 merits further investigation for efficacy in clinical DMD trials, and is relevant to a diverse group of disorders through shared inflammation or membrane injury molecular pathways. By focusing on DMD as an initial indication, we benefit from having (i) models reproducing the ubiquitous molecular deficit present in all patients, and (ii) a homogenous patient population with strong foundations providing national clinical trial support. Movement into the clinic would improve treatment of human disease, provide further mechanism insight and provide a template for future drug development. With a molecular profile relevant to diverse disorders and DMD amenable to neonatal screening, VBP15 may provide an excellent opportunity to develop an Orphan disease therapy in a way that helps larger groups of more complex disorders.

MATERIALS AND METHODS

NF-κB inhibition

C2C12 cells stably expressing an NF-κB luciferase reporter were cultured and assayed as described previously (Baudy et al, 2009). For GR antagonist experiments, cells were treated with a constant concentration of drug (1 μM) and increasing RU-486 (Sigma) concentrations. In both experiments, cells were pretreated with drug for 1 h, stimulated with TNFα (10 ng/ml) and assayed for luciferase activity 3 h later. H2K myoblasts were cultured with gamma-interferon at 33°C, and differentiated into myotubes in six-well plates with Matrigel at 37°C. Cells were plated 1E5 per well, treated with drug after 4 days of differentiation, induced with TNFα 24 h later, and RNA harvested the following day.

Pituitary cell assays

For pituitary cell line experiments, AtT-20/D16v-F2 cells (ATCC) were maintained at 37°C, 5% humidity in DMEM with 10% FBS. In *Sgk1* studies, cells were plated at 6E5 per well overnight, then serum starved in six-well plates. After 48 h, cells were drug treated for 6 h then lysed for RNA. For ACTH studies, cells were plated at 1.3E6 per T25 flask and treated with drug. Media and drug were changed daily for 5 days, then cells were counted and replated in six-well plates. Twenty-four hours later, media was collected and cells lysed for RNA. ACTH secretion was assessed by IumELISA (Calbiotech).

GR mutant assays

GR^{null} cells and the parental L929 fibroblast line they were derived from (Housley & Forsthoefel, 1989) were cultured in DMEM at 37°C. Protein lysates were obtained from untreated cells using RIPA buffer, separated on 4–15% PAGE gels, and transferred to nitrocellulose membranes, which were immunoblotted with rabbit polyclonal anti-GR (Santa Cruz) and rabbit monoclonal anti-GAPDH (Cell Signaling Technology), followed by HRP-secondary (Bio-Rad). For assays of GRE and inflammatory transcripts, cells were treated with drug for 24 h, then stimulated with TNFα (1 ng/ml) for an additional 24 h, lysed for RNA, and assayed by qPCR.

$GR^{dim/dim}$ mice (Reichardt et al, 1998) were obtained from the Deutsches Krebsforschungszentrum (German Cancer Research Center). $GR^{dim/dim}$ and wild type control (C57BL/6) mice were maintained in an animal facility within IACUC guidelines under approved protocols. Spleens were isolated and single cell suspensions generated through homogenization and lysis of red blood cells using ACK lysis buffer (Lonza). Splenocytes were treated with drug for 24 h, then stimulated with TNFα (10 ng/ml) for another 24 h. RNA was extracted from splenocytes, with analysis of GRE and inflammatory transcripts performed by qPCR.

Real-time qPCR

cDNA was produced using the High Capacity cDNA Reverse Transcription Kit (ABI). Transcript levels were analysed via TaqMan qPCR assays (LifeTech). The following assays were used: *Sgk1*, Mm00441380_m1; *Pomc*, Mm00435874_m1; *Irf1*, Mm01288580_m1; *Cox2*, Mm03294838_g1; *Nos2*, Mm00440502_m1; *Tnfα*, Mm00443258_m1; *Il1a*, Mm00439620_m1; *Nfkbia*, Mm00477800_g1; *Il6*, Mm00446190_m1. qPCR was performed using TaqMan gene expression master mix and 18s rRNA as a normalization control (ABI).

Laser-mediated wounding of live cells

C2C12 myoblasts were pretreated with drug in growth media for 15 min. Immediately following this, cells were wounded in imaging media (Hank's Balanced Salts, 10 mM HEPES, pH 7.4) containing drug or equivalent vehicle, 2 mM Ca^{2+} and 2 μg/ml FM1-43 dye (Molecular Probes Inc.) at 37°C. Injuries were performed with a pulsed one-photon laser ('Ablate', Intelligent Imaging Innovations Inc.) and a custom built Olympus IX81 microscope (Olympus America). Wounding was performed with ablation power 116 in a $2 \times 2\ \mu m^2$ for all injuries. Cells were imaged at 2 s intervals. Initial fluorescence intensity was measured and used to normalize subsequent time points. Fluorescence intensity over time was measured within cell borders using SlideBook 5.0 (Intelligent Imaging Innovations Inc.).

Receptor binding assays

The various steroid receptors (GR, MR, ER, AR and PR) were extracted and incubated with a constant concentration of radiolabeled, high-affinity ligand. Increasing concentrations of unlabeled VBP1, VBP3,

VBP15 or high-affinity ligand controls (triamcinolone, spironolactone, 17-β-Estradiol, methyltrienolone or Promegestone) were added and the percent binding of radiolabeled ligands determined to gauge the affinity of the unlabeled competitors for the steroid receptors.

Animal care and drug dosing

Two separate *mdx* trials were performed to provide repeatability as well as contrasting treatment and phenotyping regimens. The larger "pre-symptomatic" *mdx* trial (78 mice total) is presented here as the primary trial. WT (C57BL/10ScSnJ) and *mdx* (C57BL/10ScSn-*Dmd*<*mdx*>/J) mice were obtained from Jackson Laboratory (Bar Harbor, ME). All experiments were conducted within IACUC guidelines under approved protocols. PND15 was chosen as the trial start point because it was the earliest age prednisolone could confidently be safely administered (Heine & Rowitch, 2009; Pinsky & Digeorge, 1965). At this point, mice were divided into groups of equally matched body mass, which were then blinded to both drug and genotype for subsequent phenotyping and histology experiments. Treatment groups ($n = 12$–14 per group) consisted of WT vehicle, *mdx* vehicle, *mdx* VBP15 (5, 15 or 30 mg/kg), and prednisolone (5 mg/kg). Mice received daily AM dosing via cherry syrup vehicle at 1 μl per 1 g body weight. One mouse suffered a head injury during phenotyping and was removed from subsequent experiments. No adverse effects from drug treatment were observed. Functional phenotyping was performed in 5-week-old mice. *In vivo* imaging was performed in 6–7 week old mice. At 8 weeks of age, terminal assays were performed and tissues harvested.

A separate 'adult' *mdx* trial (48 mice total) was performed during lead compound identification according to established standard operating procedures. In this smaller, open-label study, WT and *mdx* mice ($n = 8$ per group) received daily PM oral syrup vehicle, prednisolone (5 mg/kg) or VBP15 (5, 15 or 45 mg/kg). All mice were subjected to 30-min run on horizontal treadmills at 12 m/min, twice a week except during data collection to unmask the mild phenotype of *mdx* mice. One death was recorded at VBP15 45 mg/kg body weight. Mice were administered drug for 4 months starting at 6 weeks of age.

Motor function

At 5 weeks of age, mice in the neonate trial were assayed for motor function via grip strength measurement. Strength was assessed daily AM for 5 days using a grip strength meter (Columbus Instruments). Data was interpreted as maximum daily values for each of five testing days and averaged over the 5 days. Animals were acclimated for 1 week prior to data collection.

Live imaging

Mice were anaesthetized with isoflurane, and cathepsin caged near-infrared imaging was performed on 6–8 mice per group as described previously (Baudy et al, 2011). Briefly, mice received intraperitoneal (IP) injections of ProSense 680 (Perkin–Elmer) in PBS 24 h prior to imaging within an Optix MX2 Imager (ART). Scans of uninjected mice were performed to obtain baseline optical intensity measurements. Forelimb and hindlimb measurements were made at 0.5 mm resolution and analysed using Optiview software.

Ex vivo force contractions

At trial endpoint, EDL muscle was isolated from live anaesthetized mice and placed in Ringer's solution (137 mM NaCl, 24 mM $NaHCO_3$, 1 mM glucose, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4 and 0.025 mM tubocurarine chloride) at 25°C bubbled with 95% O_2 and 5% CO_2. Contractile properties were measured *ex vivo* according to established methods (Brooks & Faulkner, 1988) using a force apparatus (model 305B, Aurora Scientific). Drop in force was measured after 10 lengthening contractions where each muscle was stretched over 10% of its length.

Immunotoxicity studies

Peripheral blood was obtained via retro-orbital bleed. Following sacrifice, spleens and thymuses were harvested, weighed, and processed to generate single cell suspensions of splenocytes and thymocytes, respectively. Red blood cells in splenocytes and peripheral blood were lysed with 3% acetic acid + methylene blue (Stem Cell Technologies). All leucocytes were quantified via haemocytometer. For lympho-phenotyping studies, splenocytes were stained for FACS with FITC-conjugated anti-mouse CD4, PE-conjugated anti-mouse CD8, or APC-conjugated anti-mouse B220 monoclonal antibodies (eBioscience). For $CD4^+$ cell activation studies, splenocytes ($5E^5$ per well) were stimulated in RPMI 1640+ 10% FBS with 5 μg/ml concanavalin A (Sigma–Aldrich) in 48-well plates for 72 h at 37°C. Following stimulation, cells were stained with FITC-conjugated anti-mouse CD4 and APC-conjugated anti-mouse CD25 monoclonal antibodies (eBioscience). All FACS analyses were conducted using a FACSCalibur (BD Biosciences).

Histology

Paraffin cross-sections were made of gastrocnemius, heart and diaphragm muscles and stained with H&E. For gastrocnemius, images were analysed in Image J software (NIH) according to previously established methods (Spurney et al, 2009). For diaphragm, full tissue sections were scored for inflammation by a trained veterinary immunologist blinded to drug and genotype.

To assay fibrosis, paraffin embedded muscles were cross-sectioned and stained with Sirius Red. Tissue was imaged with a 4× objective, digital captures were made with Olympus software, and fibrotic signal quantified using Image J (NIH). Blood and background were removed from blinded images to prevent false detection of tissue and percent signals when threshold measurements were made during ImageJ quantitative analysis. The percentage fibrotic tissue was calculated as area reaching Sirius Red positive thresholds divided by total tissue area of the section.

X-ray and microCT analysis of bone

Skeletons were harvested at trial endpoint and stored in 10% formalin. X-rays of tibias were obtained using a Cabinet X-Ray System (Faxitron Model 43855) with exposure at 50 kVp for 1.5 min. Magnification error was calculated to be ±0.02 mm. Images were scanned and tibia lengths measured in Adobe Illustrator (v6.0) at 2400% zoom. Measurements of the opposite tibia were also obtained physically with digital calipers during dissection, with results in agreement between methods. MicroCT analysis was performed on harvested femurs using a SkyScan 1172 MicroCT (Bruker, Belgium). Imaging was performed at 40 kV source voltage, 250 uA source current, 295 ms exposure time, and 0.4° rotation step, with a 0.5 mm aluminum filter. The imaging resolution size was 6.2 um. Three-dimensional reconstructions were performed with Skyscan NRecon and Dataviewer software. Trabecular bone was

The paper explained

PROBLEM:

Glucocorticoids have been a mainstay in medicine since their discovery over 60 years ago. They are powerful anti-inflammatory drugs used to treat a variety of conditions. However, due to a complex mechanism profile, glucocorticoids also cause harsh side effects such as brittle bones, muscle wasting, stunted growth, adrenal suppression and weight gain. Patients and doctors must therefore manage their net positive and negative effects. This is of particular importance in some chronic or paediatric disorders, where lifelong treatment is required and patients must live with serious side effects. DMD is a lethal genetic muscle disease for which glucocorticoids are the current standard of care. Though glucocorticoids produce established improvements in DMD patient outcome measures, their harsh side effects dramatically affect patients' quality of life. As a result, physicians typically delay treatment in young children until well after disease onset, and many families choose to stop treatment even though there is no alternative currently available in the clinic.

RESULTS:

The discovery that glucocorticoids possess several distinct sub-activities provides an intriguing opportunity to produce drugs that stimulate some of these activities while avoiding others. We discover VBP15 as a novel, orally administered compound that shares specific anti-inflammatory effects with glucocorticoids and also acts to stabilize cell membranes. Importantly, we also find that VBP15 avoids specific activities established to cause glucocorticoid side effects. Translating these findings into mice with muscular dystrophy, we find that both preventive and therapeutic regimens improve muscle strength and disease pathology. Further, this efficacy is displayed in the absence of hormonal, immunological and growth side effects seen in glucocorticoid treated mice.

IMPACT:

There is a clear need for improved treatments in chronic inflammatory diseases such as DMD, where safer drugs would improve quality of life and provide justification for neonatal screening. Data here confirms that small molecules can be produced which separate the sub-activities of glucocorticoids towards fulfilling this need. VBP15 is identified as the lead compound, which is actively being developed towards the clinic. Excitingly, proof-of-principle data shows that this compound provides efficacy in mice with muscular dystrophy while successfully eliminating important side effects. This provides new insight into glucocorticoid sub-activities, and demonstrates the potential to replace glucocorticoids as the standard of care for DMD as well as other chronic inflammatory diseases.

selected for analysis by a polygonal region of interest within the centre of femur, starting at 70 slices (0.43 mm) proximal from the growth plate and extending proximally 200 slices (1.23 mm) further. Trabecular measurements were obtained from 3D analysis of the selected bone using Skyscan CT-analyzer software.

Statistical analyses for animal trials

Unless otherwise noted, normality of each measurement was tested via Shapiro–Wilk normality test and normally distributed measurements were compared between *mdx* treatment groups using one-way ANOVA. Measurements that were not normally distributed were compared with a non-parametric test. For efficacy studies where *mdx* treatment comparisons showed a significant overall *p*-value, *post hoc* linear tests between each VBP15 dosage group and vehicle only were performed and resulting *p*-value adjusted for multiple testing by Sidak method. In side effect assays where comparisons showed a significant overall *p*-value, a Student's *t*-test was included between prednisolone and vehicle groups for normally distributed measurements.

Author contributions

CRH designed, performed, and managed *in vitro* experiments and the *in vivo* pre-symptomatic trial, analysed data from both trials and wrote the paper. JMD designed and performed immunology and other *in vivo* experiments, analysed/interpreted data, and is actively participating in preclinical VBP compound development. QY performed several specialized mouse imaging experiments. BCD performed dissection and immunology experiments and is actively participating in preclinical development. TH contributed to the design and interpretation of experiments, and helped to perform *in vivo* experiments. JHVM performed specialized *in vivo* muscle physiology experiments. AS, BKM, and AP performed blinded, *in vivo* experiments in the neonate trial. LS performed initial *in vitro* injury experiments, provided training, and helped to interpret data. JQ and KT performed blinded histopathology experiments. SJ and SD designed and performed *in vivo* adult preclinical trial experiments. OCR and CA designed and provided X-ray imaging, services, instruction and data processing for X-ray experiments. MC contributed to histology experiment design and provided automated imaging, blinding and randomization. HGD performed statistical analyses for the two *in vivo* trials. JKJ developed the live cell laser injury technology at CNMC, provided the core equipment/services, provided funding, and participated in experimental design and analysis. EMC and JMM are responsible for identifying and developing the VBP compounds towards clinical development. EPH provided mentorship, funding, input into experimental design, and helped

to write the paper. EKMR helped to identify VBP15, is actively involved in preclinical development, contributed to data analysis/interpretation, provided mentorship and participated in trial and experimental design. KN contributed greatly to experimental designs, provided mentorship, funding, data analysis/interpretation, facilitated the *in vivo* preclinical trials, and helped to write the paper.

Acknowledgements

The authors would like to thank Brenda Klaunberg and Danielle Donahue along with the NIH Mouse Imaging Facility and Drs. Carsten Bonnemann and Jachinta Rooney for a microCT collaboration. We also thank Dr. Gregory Cox and Jackson Laboratories for assistance with *mdx* mice. GR^{null} fibroblasts were generously donated to us by Dr. Paul Housley. Drs. Alyson Fiorillo and Aurelia Defour, along with Amanda Mullen, Rana Shehata and Beryl Ampong, provided training, technical support and/or supportive efforts for work in the manuscript. X-ray imaging was performed in the Georgetown-Lombardi Preclinical Imaging Research Laboratory. Sinq systems provided automated histology imaging and randomization services. Caliper Life Sciences performed receptor binding assays. These studies were funded in part by the United States Department of Defense CDMRP grants (W81XWH-05-1-0616, W81XWH-09-1-0218, W81XWH-11-1-0754), the Foundation to Eradicate Duchenne, the Muscular Dystrophy Association USA (MDA-VP program), and the National Institutes of Health (R01-AR050478, 1U54HD053177-01A1 [Wellstone Muscular Dystrophy Center], RO1AR055686, and NCATS TRND program). Core support was received from NIH P50AR060836 (Center of Research Translation), 2R24HD050846-06 (Center for Medical Rehabilitation) and P30HD040677 (Intellectual and Developmental Disabilities Research Center). CH is funded by a T32 postdoctoral training grant in the Genetics and Genomics of Muscle (5T32AR056993-02). KN is also supported by NIH K26OD011171, the MDA (translational grant), the US Department of Defense (W81XWH-05-1-0659, W81XWH-11-1-0782), and a pilot grant from Parent Project Muscular Dystrophy. Funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Supporting Information is available at EMBO Molecular Medicine Online.

Conflict of interest statement: ReveraGen Biopharma owns method of use intellectual property relating to use of Δ-9,11 compounds to treat disease. EMC is CEO of ReveraGen. JMM, EMC, EPH and KN are co-founders of ReveraGen with shares in the company. EKMR, JMD and BCD are employees of ReveraGen.

For more information

Muscular Dystrophy Association:
http://mda.org/

Foundation to Eradicate Duchenne:
http://duchennemd.org/

Parent Project Muscular Dystrophy:
http://www.parentprojectmd.org/

Cooperative International Neuromuscular Research Group:
http://www.cinrgresearch.org/

On preclinical DMD models:
http://www.treat-nmd.eu/research/preclinical/dmd-models/

On standardized protocols for DMD model research:
http://www.treat-nmd.eu/research/preclinical/dmd-sops/

References

Aoki Y, Nakamura A, Yokota T, Saito T, Okazawa H, Nagata T, Takeda S (2010) In-frame dystrophin following exon 51-skipping improves muscle pathology and function in the exon 52-deficient mdx mouse. Mol Ther 18: 1995-2005

Avioli LV (1993) Glucocorticoid effects on statural growth. Br J Rheumatol 32: 27-30

Bansal D, Miyake K, Vogel SS, Groh S, Chen CC, Williamson R, McNeil PL, Campbell KP (2003) Defective membrane repair in dysferlin-deficient muscular dystrophy. Nature 423: 168-172

Baudy AR, Saxena N, Gordish H, Hoffman EP, Nagaraju K (2009) A robust in vitro screening assay to identify NF-kappaB inhibitors for inflammatory muscle diseases. Int Immunopharmacol 9: 1209-1214

Baudy AR, Sali A, Jordan S, Kesari A, Johnston HK, Hoffman EP, Nagaraju K (2011) Non-invasive optical imaging of muscle pathology in mdx mice using cathepsin caged near-infrared imaging. Mol Imaging Biol 13: 462-470

Baudy AR, Reeves EK, Damsker JM, Heier C, Garvin LM, Dillingham BC, McCall J, Rayavarapu S, Wang Z, Vandermeulen JH, *et al* (2012) Delta-9,11 modification of glucocorticoids dissociates nuclear factor-kappaB inhibitory efficacy from glucocorticoid response element-associated side effects. J Pharmacol Exp Ther 343: 225-232

Bauer R, Straub V, Blain A, Bushby K, MacGowan GA (2009) Contrasting effects of steroids and angiotensin-converting-enzyme inhibitors in a mouse model of dystrophin-deficient cardiomyopathy. Eur J Heart Fail 11: 463-471

Bazan NG, Marcheselli VL, Cole-Edwards K (2005) Brain response to injury and neurodegeneration: endogenous neuroprotective signaling. Ann N Y Acad Sci 1053: 137-147

Bertorini TE, Bhattacharya SK, Palmieri GM, Chesney CM, Pifer D, Baker B (1982) Muscle calcium and magnesium content in Duchenne muscular dystrophy. Neurology 32: 1088-1092

Bircan Z, Soran M, Yildirim I, Dogan M, Sahin A, Bilici A, Danaci M (1997) The effect of alternate-day low dose prednisolone on bone age in children with steroid dependent nephrotic syndrome. Int Urol Nephrol 29: 357-361

Brooks SV, Faulkner JA (1988) Contractile properties of skeletal muscles from young, adult and aged mice. J Physiol 404: 71-82

Burkin DJ, Wuebbles RD (2012) A molecular bandage for diseased muscle. Sci Transl Med 4: 139fs119

Buttgereit F, Brand MD, Burmester GR (1999) Equivalent doses and relative drug potencies for non-genomic glucocorticoid effects: a novel glucocorticoid hierarchy. Biochem Pharmacol 58: 363-368

Cai D, Frantz JD, Tawa NE, Jr, Melendez PA, Oh BC, Lidov HG, Hasselgren PO, Frontera WR, Lee J, Glass DJ, *et al* (2004) IKKbeta/NF-kappaB activation causes severe muscle wasting in mice. Cell 119: 285-298

Chase TH, Cox GA, Burzenski L, Foreman O, Shultz LD (2009) Dysferlin deficiency and the development of cardiomyopathy in a mouse model of limb-girdle muscular dystrophy 2B. Am J Pathol 175: 2299-2308

Chen YW, Nagaraju K, Bakay M, McIntyre O, Rawat R, Shi R, Hoffman EP (2005) Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. Neurology 65: 826-834

Dahlman-Wright K, Wright A, Gustafsson JA, Carlstedt-Duke J (1991) Interaction of the glucocorticoid receptor DNA-binding domain with DNA as a dimer is mediated by a short segment of five amino acids. J Biol Chem 266: 3107-3112

Deconinck AE, Rafael JA, Skinner JA, Brown SC, Potter AC, Metzinger L, Watt DJ, Dickson JG, Tinsley JM, Davies KE (1997) Utrophin-dystrophin-deficient mice as a model for Duchenne muscular dystrophy. Cell 90: 717-727

Delfin DA, Xu Y, Peterson JM, Guttridge DC, Rafael-Fortney JA, Janssen PM (2011) Improvement of cardiac contractile function by peptide-based inhibition of NF-kappaB in the utrophin/dystrophin-deficient murine model of muscular dystrophy. J Transl Med 9: 68

Diamond MI, Miner JN, Yoshinaga SK, Yamamoto KR (1990) Transcription factor interactions: selectors of positive or negative regulation from a single DNA element. Science 249: 1266-1272

Disatnik MH, Dhawan J, Yu Y, Beal MF, Whirl MM, Franco AA, Rando TA (1998) Evidence of oxidative stress in mdx mouse muscle: studies of the pre-necrotic state. J Neurol Sci 161: 77-84

Drouin J, Sun YL, Chamberland M, Gauthier Y, De Lean A, Nemer M, Schmidt TJ (1993) Novel glucocorticoid receptor complex with DNA element of the hormone-repressed POMC gene. EMBO J 12: 145-156

Dumonceaux J, Marie S, Beley C, Trollet C, Vignaud A, Ferry A, Butler-Browne G, Garcia L (2010) Combination of myostatin pathway interference and dystrophin rescue enhances tetanic and specific force in dystrophic mdx mice. Mol Ther 18: 881-887

Gajic O, Lee J, Doerr CH, Berrios JC, Myers JL, Hubmayr RD (2003) Ventilator-induced cell wounding and repair in the intact lung. Am J Respir Crit Care Med 167: 1057-1063

Gehrig SM, van der Poel C, Sayer TA, Schertzer JD, Henstridge DC, Church JE, Lamon S, Russell AP, Davies KE, Febbraio MA, *et al* (2012) Hsp72 preserves muscle function and slows progression of severe muscular dystrophy. Nature 484: 394-398

Goyenvalle A, Babbs A, Powell D, Kole R, Fletcher S, Wilton SD, Davies KE (2010) Prevention of dystrophic pathology in severely affected dystrophin/utrophin-deficient mice by morpholino-oligomer-mediated exon-skipping. Mol Ther 18: 198-205

Grady RM, Merlie JP, Sanes JR (1997a) Subtle neuromuscular defects in utrophin-deficient mice. J Cell Biol 136: 871-882

Grady RM, Teng H, Nichol MC, Cunningham JC, Wilkinson RS, Sanes JR (1997b) Skeletal and cardiac myopathies in mice lacking utrophin and dystrophin: a model for Duchenne muscular dystrophy. Cell 90: 729-738

Grady RM, Grange RW, Lau KS, Maimone MM, Nichol MC, Stull JT, Sanes JR (1999) Role for alpha-dystrobrevin in the pathogenesis of dystrophin-dependent muscular dystrophies. Nat Cell Biol 1: 215-220

Granchelli JA, Avosso DL, Hudecki MS, Pollina C (1996) Cromolyn increases strength in exercised mdx mice. Res Commun Mol Pathol Pharmacol 91: 287-296

Griggs RC, Moxley RT, III, Mendell JR, Fenichel GM, Brooke MH, Pestronk A, Miller JP, Cwik VA, Pandya S, Robison J, *et al* (1993) Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). Neurology 43: 520-527

Grounds MD, Shavlakadze T (2011) Growing muscle has different sarcolemmal properties from adult muscle: a proposal with scientific and clinical implications: reasons to reassess skeletal muscle molecular dynamics, cellular responses and suitability of experimental models of muscle disorders. Bioessays 33: 458-468

Grounds MD, Torrisi J (2004) Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis. FASEB J 18: 676-682

Guo C, Willem M, Werner A, Raivich G, Emerson M, Neyses L, Mayer U (2006) Absence of alpha 7 integrin in dystrophin-deficient mice causes a myopathy similar to Duchenne muscular dystrophy. Hum Mol Genet 15: 989-998

Heine VM, Rowitch DH (2009) Hedgehog signaling has a protective effect in glucocorticoid-induced mouse neonatal brain injury through an 11betaHSD2-dependent mechanism. J Clin Invest 119: 267-277

Hoffman EP, Bronson A, Levin AA, Takeda S, Yokota T, Baudy AR, Connor EM (2011) Restoring dystrophin expression in duchenne muscular dystrophy muscle progress in exon skipping and stop codon read through. Am J Pathol 179: 12-22

Housley PR, Forsthoefel AM (1989) Isolation and characterization of a mouse L cell variant deficient in glucocorticoid receptors. Biochem Biophys Res Commun 164: 480-487

Howard AC, McNeil AK, McNeil PL (2011) Promotion of plasma membrane repair by vitamin E. Nat Commun 2: 597

Itani OA, Liu KZ, Cornish KL, Campbell JR, Thomas CP (2002) Glucocorticoids stimulate human sgk1 gene expression by activation of a GRE in its 5′-flanking region. Am J Physiol Endocrinol Metab 283: E971-E979

Jaiswal JK, Marlow G, Summerill G, Mahjneh I, Mueller S, Hill M, Miyake K, Haase H, Anderson LV, Richard I, *et al* (2007) Patients with a non-dysferlin Miyoshi myopathy have a novel membrane repair defect. Traffic 8: 77-88

Johnsen SD (2001) Prednisone therapy in Becker's muscular dystrophy. J Child Neurol 16: 870-871

Kar NC, Pearson CM (1978) Muscular dystrophy and activation of proteinases. Muscle Nerve 1: 308-313

Kavanagh RJ, Kam PC (2001) Lazaroids: efficacy and mechanism of action of the 21-aminosteroids in neuroprotection. Br J Anaesth 86: 110-119

Kissel JT, Lynn DJ, Rammohan KW, Klein JP, Griggs RC, Moxley RT, III, Cwik VA, Brooke MH, Mendell JR (1993) Mononuclear cell analysis of muscle biopsies in prednisone- and azathioprine-treated Duchenne muscular dystrophy. Neurology 43: 532-536

Knuesel I, Riban V, Zuellig RA, Schaub MC, Grady RM, Sanes JR, Fritschy JM (2002) Increased vulnerability to kainate-induced seizures in utrophin-knockout mice. Eur J Neurosci 15: 1474-1484

Landis SC, Amara SG, Asadullah K, Austin CP, Blumenstein R, Bradley EW, Crystal RG, Darnell RB, Ferrante RJ, Fillit H, *et al* (2012) A call for transparent reporting to optimize the predictive value of preclinical research. Nature 490: 187-191

Lipworth BJ (2000) Therapeutic implications of non-genomic glucocorticoid activity. Lancet 356: 87-89

Manolagas SC, Weinstein RS (1999) New developments in the pathogenesis and treatment of steroid-induced osteoporosis. J Bone Miner Res 14: 1061-1066

Marques MJ, Ventura Machado R, Minatel E, Santo Neto H (2008) Disodium cromoglycate protects dystrophin-deficient muscle fibers from leakiness. Muscle Nerve 37: 61-67

Megeney LA, Kablar B, Perry RL, Ying C, May L, Rudnicki MA (1999) Severe cardiomyopathy in mice lacking dystrophin and MyoD. Proc Natl Acad Sci USA 96: 220-225

Meijsing SH, Pufall MA, So AY, Bates DL, Chen L, Yamamoto KR (2009) DNA binding site sequence directs glucocorticoid receptor structure and activity. Science 324: 407-410

Mourkioti F, Kratsios P, Luedde T, Song YH, Delafontaine P, Adami R, Parente V, Bottinelli R, Pasparakis M, Rosenthal N (2006) Targeted ablation of IKK2 improves skeletal muscle strength, maintains mass, and promotes regeneration. J Clin Invest 116: 2945-2954

Nagaraju K, Willmann R (2009) Developing standard procedures for murine and canine efficacy studies of DMD therapeutics: report of two expert workshops on "Pre-clinical testing for Duchenne dystrophy": Washington DC, October 27th–28th 2007 and Zurich, June 30th–July 1st 2008. Neuromuscul Disord 19: 502-506

Peterson JM, Kline W, Canan BD, Ricca DJ, Kaspar B, Delfin DA, DiRienzo K, Clemens PR, Robbins PD, Baldwin AS, *et al* (2011) Peptide-based inhibition of NF-kappaB rescues diaphragm muscle contractile dysfunction in a murine model of Duchenne muscular dystrophy. Mol Med 17: 508-515

Pinsky L, Digeorge AM (1965) Cleft palate in the mouse: a teratogenic index of glucocorticoid potency. Science 147: 402-403

Porter JD, Khanna S, Kaminski HJ, Rao JS, Merriam AP, Richmonds CR, Leahy P, Li J, Guo W, Andrade FH (2002) A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. Hum Mol Genet 11: 263-272

Porter JD, Merriam AP, Leahy P, Gong B, Khanna S (2003) Dissection of temporal gene expression signatures of affected and spared muscle groups in dystrophin-deficient (mdx) mice. Hum Mol Genet 12: 1813-1821

Rando TA, Disatnik MH, Yu Y, Franco A (1998) Muscle cells from mdx mice have an increased susceptibility to oxidative stress. Neuromuscul Disord 8: 14-21

Raymackers JM, Debaix H, Colson-Van Schoor M, De Backer F, Tajeddine N, Schwaller B, Gailly P, Gillis JM (2003) Consequence of parvalbumin deficiency in the mdx mouse: histological, biochemical and mechanical phenotype of a new double mutant. Neuromuscul Disord 13: 376-387

Reeves EK, Hoffman EP, Nagaraju K, Damsker JM, McCall JM (2013) VBP15: preclinical characterization of a novel anti-inflammatory delta 9,11 steroid. Bioorg Med Chem 21: 2241-2249

Reichardt HM, Kaestner KH, Tuckermann J, Kretz O, Wessely O, Bock R, Gass P, Schmid W, Herrlich P, Angel P, *et al* (1998) DNA binding of the glucocorticoid receptor is not essential for survival. Cell 93: 531-541

Rhen T, Grissom S, Afshari C, Cidlowski JA (2003) Dexamethasone blocks the rapid biological effects of 17beta-estradiol in the rat uterus without antagonizing its global genomic actions. FASEB J 17: 1849-1870

Saija A, Tomaino A, Pellegrino ML, Giuffrida N, Trombetta D, Castelli F (2001) In vitro evaluation of the antioxidant activity and biomembrane interaction of the lazaroid U-74389G. Life Sci 68: 1351-1366

Sali A, Guerron AD, Gordish-Dressman H, Spurney CF, Iantorno M, Hoffman EP, Nagaraju K (2012) Glucocorticoid-treated mice are an inappropriate positive control for long-term preclinical studies in the mdx mouse. PLoS ONE 7: e34204

Sharma N, Medikayala S, Defour A, Rayavarapu S, Brown KJ, Hathout Y, Jaiswal JK (2012) Use of quantitative membrane proteomics identifies a novel role of mitochondria in healing injured muscles. J Biol Chem 287: 30455-30467

Shivaji S, Jagannadham MV (1992) Steroid-induced perturbations of membranes and its relevance to sperm acrosome reaction. Biochim Biophys Acta 1108: 99-109

Spurney CF, Gordish-Dressman H, Guerron AD, Sali A, Pandey GS, Rawat R, Van Der Meulen JH, Cha HJ, Pistilli EE, Partridge TA, *et al* (2009) Preclinical drug trials in the mdx mouse: assessment of reliable and sensitive outcome measures. Muscle Nerve 39: 591-602

Spurney CF, Guerron AD, Yu Q, Sali A, van der Meulen JH, Hoffman EP, Nagaraju K (2011) Membrane sealant Poloxamer P188 protects against isoproterenol induced cardiomyopathy in dystrophin deficient mice. BMC Cardiovasc Disord 11: 20

Takeda A, Jimi T, Wakayama Y, Misugi N, Miyake S, Kumagai T (1992) Demonstration of cathepsins B, H and L in xenografts of normal and Duchenne-muscular-dystrophy muscles transplanted into nude mice. Biochem J 288: 643-648

Townsend D, Turner I, Yasuda S, Martindale J, Davis J, Shillingford M, Kornegay JN, Metzger JM (2010) Chronic administration of membrane sealant prevents severe cardiac injury and ventricular dilatation in dystrophic dogs. J Clin Invest 120: 1140-1150

Wakefield PM, Tinsley JM, Wood MJ, Gilbert R, Karpati G, Davies KE (2000) Prevention of the dystrophic phenotype in dystrophin/utrophin-deficient muscle following adenovirus-mediated transfer of a utrophin minigene. Gene Ther 7: 201-204

Weisleder N, Takizawa N, Lin P, Wang X, Cao C, Zhang Y, Tan T, Ferrante C, Zhu H, Chen PJ, *et al* (2012) Recombinant MG53 protein modulates therapeutic cell membrane repair in treatment of muscular dystrophy. Sci Transl Med 4: 139ra185

Willmann R, Possekel S, Dubach-Powell J, Meier T, Ruegg MA (2009) Mammalian animal models for Duchenne muscular dystrophy. Neuromuscul Disord 19: 241-249

Willmann R, De Luca A, Benatar M, Grounds M, Dubach J, Raymackers JM, Nagaraju K (2012) Enhancing translation: guidelines for standard preclinical experiments in mdx mice. Neuromuscul Disord 22: 43-449

Wissink S, van Heerde EC, Schmitz ML, Kalkhoven E, van der Burg B, Baeuerle PA, van der Saag PT (1997) Distinct domains of the RelA NF-kappaB subunit are required for negative cross-talk and direct interaction with the glucocorticoid receptor. J Biol Chem 272: 22278-22284

Wolthers OD, Pedersen S (1990) Short term linear growth in asthmatic children during treatment with prednisolone. BMJ 301: 145-148

Yasuda S, Townsend D, Michele DE, Favre EG, Day SM, Metzger JM (2005) Dystrophic heart failure blocked by membrane sealant poloxamer. Nature 436: 1025-1029

Yoshiuchi I, Shingu R, Nakajima H, Hamaguchi T, Horikawa Y, Yamasaki T, Oue T, Ono A, Miyagawa JI, Namba M, *et al* (1998) Mutation/polymorphism scanning of glucose-6-phosphatase gene promoter in noninsulin-dependent diabetes mellitus patients. J Clin Endocrinol Metab 83: 1016-1019

Published in final edited form as:
Phys Med Rehabil Clin N Am. 2012 November ; 23(4): 821–828. doi:10.1016/j.pmr.2012.08.003.

Novel Approaches to Corticosteroid Treatment in Duchenne Muscular Dystrophy

Eric P. Hoffman, Ph.D.[a,b,*], **Erica Reeves, Ph.D.**[b], **Jesse Damsker, Ph.D.**[b], **Kanneboyina Nagaraju, D.V.M., Ph.D.**[a,b], **John M. McCall, Ph.D.**[b], **Edward M. Connor, M.D.**[a,b], and **Kate Bushby, M.D.**[c] **for the FOR DMD investigators of the MSG and Treat NMD**

[a]Center for Genetic Medicine Research, Children's National Medical Center, 111 Michigan Ave. NW, Washington, DC, 20010, USA

[b]ReveraGen Biopharma, 9700 Great Seneca Hwy, Rockville, MD 20850, USA

[c]Institute of Human Genetics, International Centre for Life, Central Parkway, Newcastle upon Tyne NE1 3BZ, UK

Keywords

Duchenne muscular dystrophy; Corticosteroids; Glucocorticoids; Dissociative steroids

INTRODUCTION

Duchenne muscular dystrophy (DMD) is an X-linked progressive muscular dystrophy, caused by loss of the dystrophin protein at the myofiber membrane.[1,2] Pharmacologic treatment of DMD patients with glucocorticoids can improve patient strength and prolong ambulation, with concomitant improvements in quality-of-life scales.[3–9] As such, glucocorticoid treatment for DMD is recommended in standard-of-care guidelines, and as an American Academy of Neurology practice parameter.[10–12] The majority of trials and treatment recommendations have used an oral dose of prednisone at 0.75 mg/kg/d. However, alternative dosing regimens have been reported as changing the efficacy versus side-effect profiles, including weekend dosing,[13,14] lower doses,[15] and alternative-day doses (10 mg/kg/wk divided over 2 weekend days).[16–18] In each study, a goal was to achieve a better balance of efficacy (increased strength and delay of disease progression) with fewer side effects (bone fragility, weight gain, mood changes).[19–21] It is pertinent to note that muscle weakness and wasting is an acknowledged side effect of chronic glucocorticoid administration in many indications, such as critical care medicine, and is the most common drug-induced form of muscle weakness.[22] Glucocorticoids have a direct molecular effect on myofibers, stimulating the catabolic AKT1/FOXO1 pathway, decreasing protein synthesis and increasing the rate of protein catabolism, resulting in weakness and atrophy.[23] Thus it is likely that DMD patients treated with glucocorticoids show the clinical outcome of increased muscle strength mitigated to some extent by the side effect of muscle catabolism. Clearly any effort to reduce side effects such as weight gain and short stature may also lead to lessening of the side effect of muscle weakness, whereby the balance would then be tipped to greater efficacy.

[*]Corresponding author. Center for Genetic Medicine Research, Children's National Medical Center, 111 Michigan Ave. NW, Washington, DC. 20010 ehoffman@cnmcresearch.org.

Fluorinated glucocorticoids, such as dexamethasone, are considerably more potent, with higher affinity to the glucocorticoid receptor (Fig. 1). However, these tend to also exacerbate side effects, and are generally avoided in indications of chronic use, such as muscular dystrophy. On the other hand, less potent nonfluorinated varieties of glucocorticoids have been tried, such as deflazacort. Deflazacort trials in DMD have suggested similar efficacy to that of prednisone (albeit at a higher dose), with an improvement in some side-effect profiles.[3,24–26]

FINDING THE OPTIMUM REGIMEN OF CORTICOSTEROIDS FOR DMD (FOR-DMD) CLINICAL TRIAL

To study the balance of efficacy and side effects, depending on steroid type (prednisone vs deflazcort) and dosing regimen (daily vs 10 days on, 10 days off), the FOR-DMD trial was designed and implemented. FOR-DMD is a multicenter, double-blind, parallel-group, 36- to 60-month study, comparing 3 corticosteroid regimens in wide use in DMD:

- Daily prednisone (0.75 mg/kg/d)
- Intermittent prednisone (0.75 mg/kg/d, 10 days on, 10 days off)
- Daily deflazacort (0.9 mg/kg/d).

The hypothesis being tested is that daily corticosteroids (prednisone or deflazacort) will be of greater benefit than intermittent corticosteroids (prednisone) in terms of function and subject/parent satisfaction. A secondary outcome is to study whether daily deflazacort will be associated with a better side-effect profile than daily prednisone.

The primary outcome variable will be a 3-dimensional (multivariate) outcome consisting of the following 3 components (each averaged over all postbaseline follow-up visits through month 36): (1) time to stand from lying (log-transformed), (2) forced vital capacity, and (3) subject/parent global satisfaction with treatment, as measured by the Treatment Satisfaction Questionnaire for medication.

Secondary outcome variables will include regimen tolerance, adverse event profile, and secondary functional outcomes including the 6-minute walk test, quality of life, and cardiac function. The analyses will be adjusted for covariates, namely country/ region, baseline time to stand from lying, baseline forced vital capacity (FVC), and initial weight band. A sample size of 100 subjects per group (300 in total) will provide adequate power to detect differences that are thought to be of minimal clinical significance between any 2 of the 3 treatment groups, assuming a 10% rate of subject withdrawal.

The trial will randomize 300 boys aged 4 to 7 years to 0.75 mg/kg/d prednisone; 0.75 mg/kg/d prednisone for 10 days alternating with 10 days off; or 0.9 mg/kg/d deflazacort. All boys will complete a minimum 3 years (36 months) treatment period. All boys entering the trial will remain on the study drug until the last boy completes the 36 months of study; this may be up to 60 months.

Eligible boys will be those with confirmed DMD (defined as male with clinical signs compatible with DMD and confirmed DMD mutation in the dystrophin gene [out-of-frame deletion or point mutation or duplication] or absent/<3% dystrophin on muscle biopsy); age at least 4 years and under 8 years; ability to rise independently from the floor; willingness and ability of parent or legal guardian to give informed consent; willingness and ability to comply with scheduled visits, drug administration plan, and study procedures; and ability to maintain reproducible FVC measurements.

The study is funded by the National Institutes of Health (Kate Bushby and Robert Griggs, study Chairs), and will begin enrollment in 2012.

DEVELOPMENT OF DISSOCIATIVE STEROIDS FOR DMD

An alternative approach to optimizing dosing regimens of traditional glucocorticoid drugs is to change the chemistry of the drug, with the goal of broadening the therapeutic window (increasing efficacy while decreasing side effects). Glucocorticoid drugs are recognized to have 2 subactivities: serving as a ligand for steroid hormone receptors, and nonreceptor-mediated effects on plasma membranes. The ligand/ receptor complex has 2 further subactivities: transactivation and transrepression properties. Transactivation (also termed *cis*-regulation) is the best characterized molecular response, whereby ligand/glucocorticoid receptor complexes translocate from the cytoplasm to the nucleus and then interact directly with DNA and gene promoters (Fig. 2). With transactivation, the ligand/receptor dimers typically bind to a DNA sequence motif (glucocorticoid response elements [GRE]), and activate transcription of the nearby gene (hence the designation “transactivation”). Of importance, there is increasing evidence that the transactivation subactivity is associated more with side effects rather than with drug efficacy.[27]

Clinical efficacy, on the other hand, is increasingly associated with the second, trans-repression subactivity. Transrepression involves ligand/receptor interactions with other cellular signaling proteins, such as nuclear factor (NF)-κB, activator protein 1, and STAT5 complexes, with downstream changes in cell signaling, and more indirect effects on gene transcription (non–GRE-mediated).[27,28] Transrepression has been associated with anti-inflammatory activity and clinical efficacy.

All steroid hormones, including glucocorticoids, are lipophilic, and readily traverse lipid bilayers (cell membranes). Some steroid drugs have been optimized for membrane activities, such as the lazaroids (see Fig. 1). Lazaroids, including the Δ-9,11 modification thought to block binding of the drug to the receptor, were optimized for effects on cell membranes (prevention of lipid peroxidation), and tested clinically for neuroprotection.[29–31] In DMD, there are well-documented changes in myofiber membrane function and integrity, and steroids are likely to modify this defect (for better or worse). Consistent with this, recent studies of lazaroids in myogenic cells in culture[32] and ischemia/reperfusion injury in vivo have shown benefit of lazaroid drugs.[33]

In an effort to improve upon glucocorticoid therapy for DMD, the authors studied drugs with the Δ-9,11 chemistry (see Fig. 1). The goal was to determine whether this chemistry represented a dissociative steroid (eg, separation of the transactivation [side effects] and transrepression [efficacy]) (see Fig. 2). A Δ-9,11 drug, anecortave, did in fact bind the glucocorticoid receptor, albeit at lower affinity than pharmacologic glucocorticoids.[34] Of importance, the ligand/glucocorticoid receptor complex was found to translocate to the nucleus, but showed no activity in binding to GRE elements and activating GRE-mediated gene transcription. Thus, the Δ-9,11 drug appeared to have lost the transactivation subactivity associated with many deleterious side effects (see Fig. 2).

To determine whether the Δ-9,11 drug retained transrepression (the subactivity associated with glucocorticoid efficacy), the authors studied anti-inflammatory effects using NF-κB reporter assays.[35] NF-κB inhibitory activity was found to be retained by the Δ-9,11 drug, at a potency similar to that of prednisone.[34] We were also interested in the effects of the drugs on the phospholipids that make up the membrane. Phospholipid bilayers have a hydrophilic head on the inner and outer walls of the membrane and hydrophobic tails. Lipids such as cholesterol are known to compress head groups, strengthen the bilayer, and decrease permeability when incorporated into a lipid bilayer. Indeed, our delta 9,11 steroids exert a

similar and more profound effect on phospholipid bilayers than either prednisolone or cholesterol. The D-ring functionality (17-hydroxy-20-keto-21-hydroxy) orients within the phospholipid head groups while the hydrophobic ABC and most of the D ring orients in the lipid core. Since the C-17 C-20 bond can rotate, our compounds are operationally cone-like wedges in the phospholipid. They compress the head groups and decrease permeability while disordering the hydrophobic core which, among other things, protects against lipid peroxidation by decreasing the repeat number in lipid peroxidation chain reactions. This phenomenon for the delta 9,11 steroids has been described.[36] These result encouraged the authors to conduct a preclinical study of the dystrophin-deficient *mdx* mouse model of DMD. Evidence of efficacy in vivo was found, whereby daily oral delivery of Δ-9,11 analogue reduced muscle inflammation and improved multiple functional assays. Of note, no side effects of reductions in body weight or spleen size seen with prednisone treatment were observed, suggesting that the Δ-9,11 drug had indeed lost side effects. These data suggest that the Δ-9,11 chemistry holds promise as a dissociative steroid, with retention of efficacy via transrepression, and loss of side effects via reductions in transactivation subactivities. Current studies are focused on testing a series of Δ-9,11 compounds to optimize the potency, bioavailability, and toxicity profiles (lead compound selection), as well as testing of the optimized lead compound in animal models of multiple chronic inflammatory conditions, including other types of muscular dystrophy.

SUMMARY

DMD is among the most common of the muscular dystrophies, leading to shortened life span and considerable disability. Glucocorticoids are considered the standard of care, yet dose regimens have not been optimized, and the balance of efficacy and side effects for specific types of glucocorticoids requires further study. The FOR-DMD trial promises to shed light on dose optimization, as well as the therapeutic window of prednisone versus deflazacort. An alternative approach to optimizing currently available steroid regimens is to develop new drugs that are able to broaden the therapeutic window (increased efficacy with decreased side effects). Initial studies of Δ-9,11 modifications of the steroid backbone suggests that this chemistry produces a dissociative steroid, whereby anti-inflammatory activity is retained (transrepression) and membrane stabilization properties enhanced, while side effects are mitigated (loss of transactivation subactivity). Current studies are focusing on lead compound optimization using transactivation and membrane stability assays.

Acknowledgments

The project described is supported by grant number U01NS061799 from the National Institute of Neurological Disorders and Stroke and U54HD053177 from the National Institute for Child Health and Human Development. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Neurological Disorders and Stroke, National Institute of Child Health and Human Development, or the National Institutes of Health. The authors acknowledge funding to TREAT-NMD by the EU FP6. Newcastle upon Tyne is a partner in the MRC Centre for Neuromuscular Diseases. ReveraGen receives funding from the National Institutes of Health TRND program of the National Center for Advancing Clinical Sciences, the Muscular Dystrophy Association Venture Philanthropy Fund, the US Department of Defense CDRMP, and Foundation to Eradicate Duchenne (FED).

References

1. Hoffman EP, Brown RH, Kunkel LM. Dystrophin: the protein product of the Duchenne muscular dystrophy locus. Cell. 1987; 51:919–28. [PubMed: 3319190]
2. Koenig M, Hoffman EP, Bertelson CJ, et al. Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. Cell. 1987; 50:509–17. [PubMed: 3607877]

3. Angelini C, Pegoraro E, Turella E, et al. Deflazacort in Duchenne dystrophy: study of long-term effect. Muscle Nerve. 1994; 17(4):386–91. [PubMed: 8170484]
4. Biggar WD, Harris VA, Eliasoph L, et al. Long-term benefits of deflazacort treatment for boys with Duchenne muscular dystrophy in their second decade. Neuro-muscul Disord. 2006; 16:249–55.
5. Griggs RC, Moxley RT III, Mendell JR, et al. Prednisone in Duchenne dystrophy: a randomized, controlled trial defining the time course and dose response: Clinical Investigation of Duchenne Dystrophy Group. Arch Neurol. 1991; 48:383–8. [PubMed: 2012511]
6. Griggs RC, Moxley RT III, Mendell JR, et al. Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). Neurology. 1993; 43:520–7. [PubMed: 8450994]
7. Manzur AY, Kuntzer T, Pike M, et al. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev. 2008; (1):CD003725. [PubMed: 18254031]
8. Markham LW, Kinnett K, Wong BL, et al. Corticosteroid treatment retards development of ventricular dysfunction in Duchenne muscular dystrophy. Neuromuscul Disord. 2008; 18:365–70. [PubMed: 18436445]
9. Mendell JR, Moxley RT, Griggs RC, et al. Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy. N Engl J Med. 1989; 320:1592–7. [PubMed: 2657428]
10. Moxley RT III, Ashwal S, Pandya S, et al. Practice parameter: corticosteroid treatment of Duchenne dystrophy: report of the Quality Standards Subcommittee of the American Academy of Neurology and the Practice Committee of the Child Neurology Society. Neurology. 2005; 64:13–20. [PubMed: 15642897]
11. Bushby K, Finkel R, Birnkrant DJ, et al. Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. Lancet Neurol. 2010; 9:77–93. [PubMed: 19945913]
12. Bushby K, Finkel R, Birnkrant DJ, et al. Diagnosis and management of Duchenne muscular dystrophy, part 2: implementation of multidisciplinary care. Lancet Neurol. 2010; 9:177–89. [PubMed: 19945914]
13. Connolly AM, Schierbecker J, Renna R, et al. High dose weekly oral prednisone improves strength in boys with Duchenne muscular dystrophy. Neuromuscul Disord. 2002; 12:917–25. [PubMed: 12467746]
14. Escolar DM, Hache LP, Clemens PR, et al. Randomized, blinded trial of weekend vs daily prednisone in Duchenne muscular dystrophy. Neurology. 2011; 77(5):444–52. [PubMed: 21753160]
15. Brooke MH, Fenichel GM, Griggs RC, et al. Clinical investigation of Duchenne muscular dystrophy: interesting results in a trial of prednisone. Arch Neurol. 1987; 44:812–7. [PubMed: 3632393]
16. Fenichel GM, Mendell JR, Moxley RT III, et al. A comparison of daily and alternate-day prednisone therapy in the treatment of Duchenne muscular dystrophy. Arch Neurol. 1991; 48:575–9. [PubMed: 2039377]
17. Beenakker EA, Fock JM, Van Tol MJ, et al. Intermittent prednisone therapy in Duchenne muscular dystrophy: a randomized controlled trial. Arch Neurol. 2005; 62:128–32. [PubMed: 15642859]
18. Sansome A, Royston P, Dubowitz V. Steroids in Duchenne muscular dystrophy: pilot study of a new low-dosage schedule. Neuromuscul Disord. 1993; 3:567–9. [PubMed: 8186713]
19. Matthews DJ, James KA, Miller LA, et al. MD STARnet. Use of corticosteroids in a population-based cohort of boys with Duchenne and Becker muscular dystrophy. J Child Neurol. 2010; 25(11):1319–24. [PubMed: 20207610]
20. Angelini C. The role of corticosteroids in muscular dystrophy: a critical appraisal. Muscle Nerve. 2007; 36(4):424–35. [PubMed: 17541998]
21. Schara U, Mortier J, Mortier W. Long-term steroid therapy in Duchenne muscular dystrophy-positive results versus side effects. J Clin Neuromuscul Dis. 2001; 2(4):179–83. [PubMed: 19078632]
22. Pereira RM, Freire de Carvalho J. Glucocorticoid-induced myopathy. Joint Bone Spine. 2011; 78(1):41–4. [PubMed: 20471889]

23. Hoffman EP, Nader GA. Balancing muscle hypertrophy and atrophy. Nat Med. 2004 Jun; 10(6): 584–5. [PubMed: 15170202]
24. Mesa LE, Dubrovsky AL, Corderi J, et al. Steroids in Duchenne muscular dystrophy—deflazacort trial. Neuromuscul Disord. 1991; 1(4):261–6. [PubMed: 1822804]
25. Bonifati MD, Ruzza G, Bonometto P, et al. A multicenter, double-blind, randomized trial of deflazacort versus prednisone in Duchenne muscular dystrophy. Muscle Nerve. 2000; 23(9):1344–7. [PubMed: 10951436]
26. Biggar WD, Politano L, Harris VA, et al. Deflazacort in Duchenne muscular dystrophy: a comparison of two different protocols. Neuromuscul Disord. 2004; 14(8–9):476–82. [PubMed: 15336688]
27. Newton R, Holden NS. Separating transrepression and transactivation: a distressing divorce for the glucocorticoid receptor? Mol Pharmacol. 2007; 72:799–809. [PubMed: 17622575]
28. Rhen T, Cidlowski JA. Anti-inflammatory action of glucocorticoids—new mechanisms for old drugs. N Engl J Med. 2005; 353:1711–23. [PubMed: 16236742]
29. Taylor BM, Fleming WE, Benjamin CW, et al. The mechanism of cytoprotective action of lazaroids I: Inhibition of reactive oxygen species formation and lethal cell injury during periods of energy depletion. J Pharmacol Exp Ther. 1996; 276:1224–31. [PubMed: 8786555]
30. Bracken MB, Shephard MJ, Holford TR, et al. Administration of methylpredniso-lone for 24 or 48 h or tirilazadmesylate for 48 h in the treatment of acute spinal cord injury; results of the third national acute spinal cord injury randomized controlled trial. JAMA. 1997; 277:1597–604. [PubMed: 9168289]
31. Kavanagh RJ, Kam PC. Lazaroids: efficacy and mechanism of action of the 21-aminosteroids in neuroprotection. Br J Anaesth. 2001; 86:110–9. [PubMed: 11575384]
32. Passaquin AC, Lhote P, Rüegg UT. Calcium influx inhibition by steroids and analogs in C2C12 skeletal muscle cells. Br J Pharmacol. 1998; 124:1751–9. [PubMed: 9756393]
33. Campo GM, Squadrito F, Campo S, et al. Antioxidant activity of U-83836E, a second generation lazaroid, during myocardial ischemia/reperfusion injury. Free Radic Res. 1997; 27:577–90. [PubMed: 9455693]
34. Baudy AR, Reeves E, Damsker JM, et al. Δ-9,11 modification of glucocorticoids dissociate NF-κB inhibitory efficacy from GRE-associated side effects. J Pharmacol Exp Ther. 2012; 343:225–32. [PubMed: 22743576]
35. Baudy AR, Saxena N, Gordish H, et al. A robust in vitro screening assay to identify NF-kappaB inhibitors for inflammatory muscle diseases. Int Immunopharmacol. 2009; 9:1209–14. [PubMed: 19596085]
36. Epps DE, McCall JM. Physical and Chemical Mechanisms of the Antioxidant Action of Tirilazad Mesylate. Handbook of Novel Antioxidants Antioxid Health Dis. 1997; 4:95–137.

KEY POINTS

- Current standard of care of Duchenne muscular dystrophy (DMD) includes pharmacologic treatment with oral glucocorticoids.
- Gains in strength and slowed progression of disease afforded by glucocorticoids are offset, in part, by the wide range of side effects of drug treatment.
- Dose optimization studies are limited, and new larger clinical studies are needed to best balance efficacy and side effects (therapeutic window), as are studies of glucocorticoid alternatives to prednisone.
- The FOR-DMD trial funded by the National Institutes of Health is under way to compare different dose regimens and types of glucocorticoids (prednisone, deflazacort).
- A novel dissociative steroid, a Δ-9,11 drug, is under clinical development for DMD. This drug promises to broaden the therapeutic window and reduce side-effect profiles.

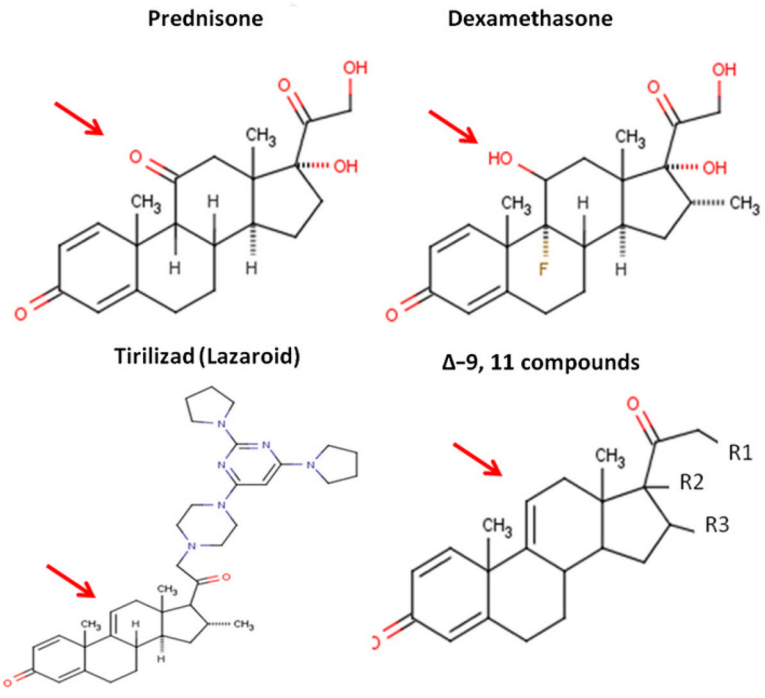


Fig. 1.

Chemical structures of glucocorticoids and dissociative steroids. The arrow indicates the position of the key 9,11 alterations distinguishing classic glucocorticoids (prednisone, dexamethasone) from dissociative steroids (Δ-9,11 analogues).

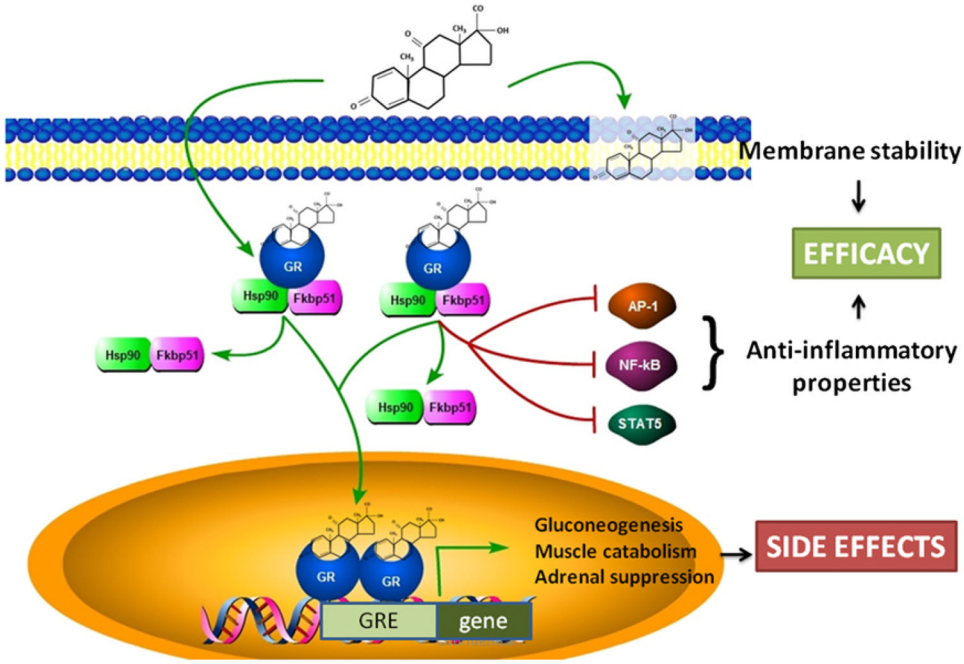


Fig. 2.

Molecular action of glucocorticoids and dissociative steroids. Classic pharmacologic glucocorticoids have anti-inflammatory, membrane fluidity, and glucocorticoid response element (GRE)-mediated transcriptional activities. Dissociative steroids retain membrane and anti-inflammatory subactivities associated with efficacy, but do not retain the GRE-mediated transcriptional activities associated with side-effect profiles. GR, glucocorticoid receptor.

AUTHOR QUERY FORM

ELSEVIER	**Journal: BMC** **Article Number: 10604**	**Please e-mail or fax your responses and any corrections to:** **E-mail: corrections.essd@elsevier.sps.co.in** **Fax: +31 2048 52799**

Dear Author,

Please check your proof carefully and mark all corrections at the appropriate place in the proof (e.g., by using on-screen annotation in the PDF file) or compile them in a separate list. Note: if you opt to annotate the file with software other than Adobe Reader then please also highlight the appropriate place in the PDF file. To ensure fast publication of your paper please return your corrections within 48 hours.

For correction or revision of any artwork, please consult http://www.elsevier.com/artworkinstructions.

Any queries or remarks that have arisen during the processing of your manuscript are listed below and highlighted by flags in the proof. Click on the 'Q' link to go to the location in the proof.

Location in article	Query / Remark: click on the Q link to go Please insert your reply or correction at the corresponding line in the proof
Q1	Please confirm that given names and surnames have been identified correctly.
Q2	Please provide more details for 'affiliation c' and check all affiliations, and correct if necessary.

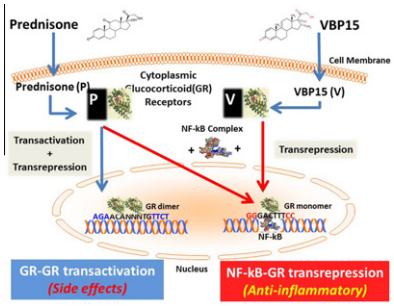
Please check this box if you have no corrections to make to the PDF file ☐

Thank you for your assistance.

Graphical abstract

VBP15: Preclinical characterization of a novel anti-inflammatory delta 9,11 steroid pp xxx–xxx

Erica K. Reeves *, Eric P. Hoffman, Kanneboyina Nagaraju, Jesse M. Damsker, John M. McCall



Bioorganic & Medicinal Chemistry xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

VBP15: Preclinical characterization of a novel anti-inflammatory delta 9,11 steroid

Q1 Erica K. Reeves[a,*], Eric P. Hoffman[a,b], Kanneboyina Nagaraju[a,b], Jesse M. Damsker[a], John M. McCall[a,c]

[a] ReveraGen BioPharma, Inc., 9700 Great Seneca Hwy Suite 160, Rockville, MD 20910, United States
[b] Center for Genetic Medicine Research, Children's National Medical Center, 111 Michigan Ave. NW, Washington, DC 20010, United States
Q2 [c] PharMac, LLC, United States

ARTICLE INFO

Article history:
Received 24 December 2012
Revised 3 February 2013
Accepted 11 February 2013
Available online xxxx

Keywords:
Glucocorticoids
VBP15
Steroids
Anti-inflammatories
Neuromuscular disease

ABSTRACT

Δ9,11 modifications of glucocorticoids (21-aminosteroids) have been developed as drugs for protection against cell damage (lipid peroxidation; lazaroids) and inhibition of neovascularization (anecortave). Part of the rationale for developing these compounds has been the loss of glucocorticoid receptor binding due to the Δ9,11 modification, thus avoiding many immunosuppressive activities and deleterious side effect profiles associated with binding to glucocorticoid and mineralocorticoid receptors. We recently demonstrated that anecortave acetate and its 21-hydroxy analog (VBP1) do, in fact, show glucocorticoid and mineralocorticoid receptor binding activities, with potent translocation of the glucocorticoid receptor to the cell nucleus. We concluded that Δ9,11 steroids showed novel anti-inflammatory properties, retaining NF-κB inhibition, but losing deleterious glucocorticoid side effect profiles. Evidence for this was developed in pre-clinical trials of chronic muscle inflammation. Here, we describe a drug development program aimed at optimizing the Δ9,11 chemistry. Twenty Δ9,11 derivatives were tested in in vitro screens for NF-κB inhibition and GR translocation to the nucleus, and low cell toxicity. VBP15 was selected as the lead compound due to potent NF-κB inhibition and GR translocation similar to prednisone and dexamethasone, lack of transactivation properties, and good bioavailability. Phamacokinetics was similar to traditional glucocorticoid drugs with terminal half-life of 0.35 h (mice), 0.58 h (rats), 5.42 h (dogs), and bioavailability of 74.5% (mice), and 53.2% (dogs). Metabolic stability showed ⩾80% remaining at 1 h of VBP6 and VBP15 in human, dog, and monkey liver microsomes. Solubility, permeability and plasma protein binding were within acceptable limits. VBP15 moderately induced CYP3A4 across the three human hepatocyte donors (24–42%), similar to other steroids. VBP15 is currently under development for treatment of Duchenne muscular dystrophy.

1. Introduction

Δ9,11 Glucocorticoids (21-aminosteroids) were designed and developed to stably incorporate into cell membranes and inhibit lipid peroxidation without glucocorticoid or mineralocorticoid activities, thus avoiding side effects associated with traditional corticosteroids.[1] A few Δ9,11 steroids brought to clinical trials were Lazaroids and Anecortave. Lazaroids have extensive hydrophilic head groups designed to optimize stability in membranes and lipid peroxidation inhibitory activities (Fig. 1). Randomized controlled trials of tirilazad mesylate in acute spinal cord injury,[2,3] and in stroke[4–6] showed good safety profiles, but failed to show clear evidence of efficacy. Pre-clinical work in additional indications continues, including multiple wound states and lung disease.[7–9]

Anecortave was developed for inhibition of neo-vascularization in age-related macular degeneration as a molecule that lacked immunomodulatory glucocorticoid activity.[10,11] The mechanism of action is thought to be through inhibition of proliferation of vascular endothelial cells, inhibition of uPA and matrix metalloproteinase 3, and stimulation of plasminogen activator inhibitor-1.[11] Controlled clinical trials of posterior juxtascleral depot (delivered at 6 month intervals) showed no safety issues, but marginal clinical benefit.[12,13] More recently, blinded controlled trials of anecortave in steroid-induced elevated intraocular pressure (IOP) have been carried out, with similar results of good safety profile.[14] The best dose of anecortave acetate showed a 31% reduction in IOP and this benefit lasted a mean duration of 56 days from a single administration.[15,16]

Previous drug development programs pursuing lazaroids and anecortave as prototype Δ9,11 glucocorticoids (21-aminosteroids) have assumed that the Δ9,11 modification inhibited drug binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor

* Corresponding author. Tel.: +1 (202) 476 1236; fax: +1 (240) 453 6208.
E-mail address: erica.reeves@reveragen.com (E.K. Reeves).

0968-0896/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.bmc.2013.02.009

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

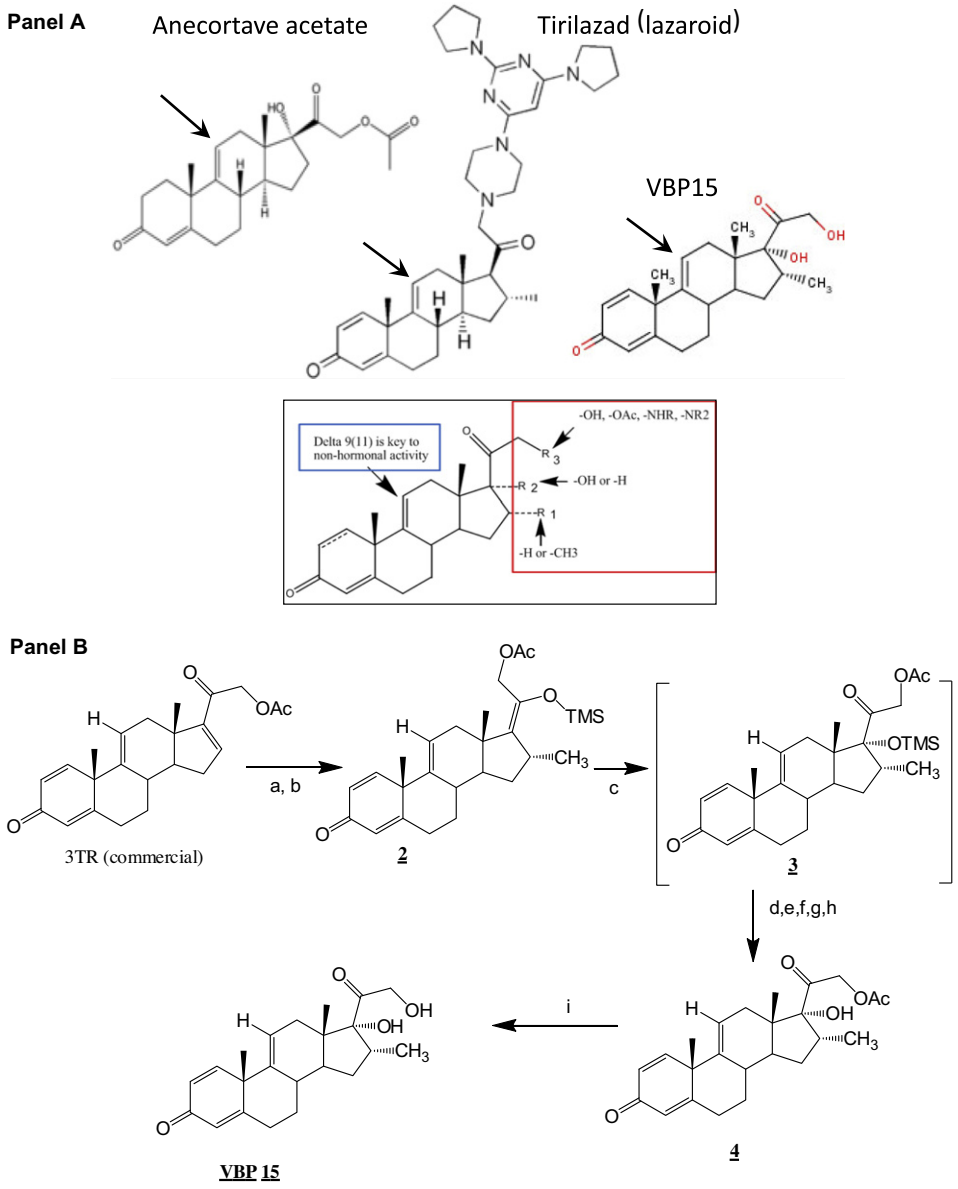


Figure 1. Structures and synthesis of Δ9,11 compounds. Panel A: Shown are two previously developed Δ9,11 steroids (Anecortave, Tirilizad), and the lead compound identified in the current study (VBP15). The Δ9,11 bond distinguishing these drugs from other steroidal compounds is shown by the black arrows. The lower panel shows the chemical modifications to the chemical backbone. The R1, R2, and R3 residues shown are those that were chemically modified with alternative chemical structures (see red box). Panel B: The synthesis of VBP15 that is pictured is typical for the synthesis of analogs in this series. (a) TMS imidazole, MeMgCl, THF; (b) $CuAc_2$, H_2O, DMPU, MeMgCl, THF; (c) peracetic acid, toluene, −10 °C; (d) $NaHSO_3$, TFA; (e) EtOAc, heptanes; (f) acetonitrile trituaration; (g) HBr, HC_2Cl_2, 40 °C; (h) MeOH crystallization; (i) K_2CO_3, MeOH, followed by HCL and crystallization from $MeOH/H_2O$.

(MR). This assumption was based largely on the lack of efficacy of the drugs in inhibiting LPS-induced inflammation in pre-clinical models—traditional glucocorticoids are impressively effective at blocking lipopolysaccharide (LPS)-induced acute inflammation.[10] However, we recently showed that both anecortave acetate and its 21-hydroxy analog (VBP1) showed relatively high affinity binding to both the GR and MR.[17] The Δ9,11 drugs induced GR translocation from the cell cytoplasm to cell nucleus, shown by both reporter and immunostaining assays, again suggesting effective ligand/receptor interactions. Nuclear translocation of steroid hormone receptors is a hallmark of classic glucocorticoid transcriptional activity, and is necessary for downstream activation of gene promoters via binding of ligand/receptor complexes to glucocorticoid response elements (GREs). However, neither Δ9,11 drug showed significant GRE-mediated transcriptional activities, either by mRNA microarray, targeted RT-PCR, or luciferase reporter constructs.[17] On the other hand, both drugs retained anti-inflammatory activity via inhibition of NF-κB pathways, and showed efficacy in inhibiting inflammation in vivo in two mouse models of chronic inflammation in muscle (dystrophin-deficient *mdx* and dysferlin-deficient *SJL* mice).[17] The discrepancy between previous findings of lack of anti-inflammatory activity, and our findings of retention of anti-inflammatory activity may be explained by the assays utilized. For example, McNatt et al. studied an acute model of LPS-induced IL-1 induction, where anecortave failed to induce IL-1, whereas glucocorticoids were effective in induction.[10] Our studies focused on in vitro assays of NF-κB inhibition and models of chronic immunity not utilizing LPS.[17]

Here, we queried the chemical space around the Δ9,11 steroid backbone, optimizing for anti-inflammatory properties (NF-κB

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

inhibition and GR nuclear translocation). The lead compound, VBP15, shows excellent drug properties, and is in development for Duchenne muscular dystrophy as the initial indication.

2. Materials and methods

2.1. Chemistry

All compounds were synthesized by Bridge Organics Co. (Kalamazoo, Michigan). Systematic (IUPAC) names: Anecortave ([2-[(8*R*,10*S*,13*S*,14*R*,17*R*)-17-hydroxy-10,13-dimethyl-3-oxo-2,6,7,8,12,14,15,16-octahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl]-2-oxo-ethyl] acetate). Tirilazad ((16α)-21-[4-(2,6-dipyrrolidin-1-ylpyrimidin-4-yl)piperazin-1-yl]-16-methylpregna-1,4,9(11)-triene-3,20-dione). Additional chemical structures studied are described in Table 1.

Traditional glucocorticoids have an 11-beta-hydroxy or an 11-keto group whereas we used a double bond between carbons 9 and 11, leading to a Δ9,11 C ring as the backbone, then developed a series of chemical entities on this backbone (Fig. 1, Panel A; Table 1). These scaffolds are not oxidatively metabolized to 11-beta hydroxy compounds, and should have less off-target effects related to active metabolites. We varied the D ring of the steroid, probing the effect of different lipophilic groups at C16 and either hydroxy or hydrogen at C17. Twenty compounds were synthesized in the VBP series of Δ9,11 compounds varying in structure at the three positions (R1, R2, R3) (Fig. 1; Table 1).

The process begins with commercially available steroid 3TR (Fig. 1; Panel B). Copper catalyzed Michael addition of MeMgCl with TMSCl through a 1,4 addition introduced a 16-alpha methyl group to give silyl enol ether 2 with 93% purity by hplc/ms and in nearly quantitative yield. The product was isolated as a toluene slurry which was oxidized with 32% by weight peracetic acid in acetic acid to yield intermediate 3 which was not isolated but was converted to compound **4** as a powder that precipitated from EtOAc/heptane trituration in 60% yield from 3TR and with 86% purity. In order to remove a small amount of the delta 9,11 epoxide, compound **4** was treated with HBr in methylene chloride. Yield from the HBr treatment after MeOH/water crystallization was 90% with 97% purity and an overall yield from 3TR of 54%. Finally, the acetate of compound **4** is removed by K_2CO_3 hydrolysis. Methanol/water crystallization of VBP15 gave a 79% yield with 98.3% purity. Overall yield from 3TR is 43%. Purity is assigned by hplc/ms. Structure was confirmed by proton and carbon NMR.

2.2. NF-κB screen

C_2C_{12} skeletal muscle cells stably transfected with a luciferase reporter construct regulated under NF-κB response element (Panomics, Fremont, CA) were used for screening NF-κB inhibitors, as we have previously described.[18] Myoblasts and myotubes grown in growth and differentiating medium respectively were pretreated with various concentrations (vehicle [DMSO]; 0.01–100 μg/ml) of the drugs for a 24 h duration before stimulating with tumor necrosis factor-α (TNF-α) (10 ng/ml) for another 24 h. After the completion of incubation, cells were washed once with PBS and lysed with cell lysis buffer to measure luciferase activity (Promega Corp, Madison, WI) using Centro LB 960 luminometer (Berthold technologies, GmbH & Co, Bad Wildbad, Germany) and EC_{50} values are calculated for each compound.

2.3. Cell viability

Cell viability was determine by MTT assay (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, St., Louis, Missouri) as per manufacturer's protocols. Percent cell viability was calculated relative to untreated cells, whereas relative luminescence units with TNF-α stimulation in the absence of drugs were considered as 100% percent. Data are represented as % inhibition relative to TNF-α induced NF-κB activation. Significance was calculated using a one-way repeated measure ANOVA to determine if there is an effect of drug concentration on cell viability.

2.4. Nuclear translocation assays

Translocation assays were performed by DiscoveRx (Fremont, CA) using GR Nuclear Translocation PathHunter cells (DiscoveRx; Fremont, CA). This assay is based on the detection of protein–protein interactions between the GR and a nuclear fusion protein containing steroid receptor co-activator peptide. The receptor is tagged with the ProLink component of enzyme fragment complementation assay system and the steroid receptor co-activator peptide (SRCP) is fused to the enzyme acceptor component. When the receptor is bound by ligand it translocates to the nucleus where it

Table 1
Chemical structures of compounds tested

VBP#	A	16	17	21	NF-κB inhibition EC_{50} (M)	Rank NF-κB	GR nuclear translocation (%)	Rank GR translocation	Sum rank
Pred/Dex					1.67E-08	1	100.00	1	2
6	Delta 1,4	CH_3	H	OH	5.64E-08	3	54.20	4	7
15	Delta 1,4	CH_3	OH	OH	6.59E-08	6	80.40	2	8
7	Delta 1,4	CH_3	H	OAc	5.41E-08	2	48.80	7	9
1	Delta 4	H	OH	OH	6.29E-08	5	49.20	6	11
16	Delta 1,4	Beta CH_3	OH	OH	6.23E-08	4	44.50	8	12
2	Delta 1,4	H	OH	OH	1.29E-07	9	57.60	3	12
3	Delta 4	H	OH	Oac	2.12E-07	10	51.50	5	15
41	Delta 1,4	Ethyl	H	OH	8.95E-08	7	27.00	11	18
17	Delta 4	CH_3	H	Dipropylamino	7.23E-07	13	37.70	9	22
11	Delta 4	H	OH	Morpholino	1.74E-06	15	28.70	10	25
5	Delta 1,4	ene	ene	OAc	9.36E-08	8	15.30	18	26
13	Delta 4	H	OH	Dipropylamino	4.35E-07	11	21.80	15	26
10	Delta 4	H	OH	Pyrrolidine	1.85E-06	16	24.70	12	28
4	Delta 1,4	ene	ene	OH	4.95E-07	12	16.70	17	29
18	Delta 4	CH_3	H	Pyrrolidine	9.88E-07	14	19.90	16	30
14	Delta 4	CH_3	H	Morpholino	5.24E-06	17	22.70	14	31
9	Delta 4	H	OH	N-Me Piperazine	5.78E-06	18	23.50	13	31
12	Delta 4	Me	H	N-Me Piperazine	>1e-5	21	14.70	19	40
59	Delta 4	*n*Butyl	H	OH	>1e-5	21	8.50	20	41
58	Delta 1,4	*n*Butyl	H	OH	>1e-5	21	6.90	21	42

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

recruits the SRCP and complementation occurs producing a chemiluminescent signal. A ten point agonist dose curve, ranging from 1×10^{-5} to 1.69×10^{-10} M was performed on all VBP compounds. Dexamethasone was performed in parallel as the standard reference control. Percentage activity is calculated using the following formula: % Activity = 100% × (Mean Relative Light Units [RLU] of test sample—mean RLU of vehicle control)/(mean MAX RLU control ligand—mean RLU of vehicle control)). EC_{50} is calculated for all the compounds.

2.5. Transactivation assays

HEK-293 cells stably transfected with a luciferase reporter construct regulated under glucocorticoid response element (GRE) (Panomics, Fremont, CA) were grown according to manufacturer's instructions. Cells were treated with various concentrations (0.01 to 100 μg/ml) of the drug for 6 h. Cells were washed once with PBS and lysed with cell lysis buffer to measure luciferase activity (Promega Corp, Madison, WI) using Centro LB 960 luminometer (Berthold technologies, GmbH & Co, Bad Wildbad, Germany).

2.6. Pharmacokinetics

Pharmacokinetic studies were carried out at Pharmaron (US headquarters 6 Venture, Suite 250, Irvine, CA 92618; work performed in Pharmaron-Beijing, 6 Taihe Road, BDA Beijing, 100176). Mice were purchased from Huafukang Bioscience, Beijing, China. Dogs were purchased from Marshall Inc., Beijing, China. Monkeys were purchased from Hainan, Inc., Jinggang, China and Guangxi Inc., Guidong China.

2.6.1. Analysis

All analyses were conducted on a Shimadzu liquid chromatograph separation system equipped with degasser DGU-20A3, solvent delivery unit LC-20AD, system controller CBM-20A, column oven CTO-10ASVP and CTC Analytics HTC PAL System. . Samples were loaded onto a Phenomenex Luna 5μ C18 (2) (2.0 × 50 mm) coupled with a preguard column with a mobile phase of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water. Mass spectrometric analysis was performed using an API 4000 instrument from AB Inc (Canada) with an ESI interfaceThe data acquisition and control system were created using Analyst 1.5 software from ABI Inc.

2.6.2. Mouse

CD1 mice ($n = 3$/drug) were injected via tail vein with a solution of 10 mg/kg VBP6 (10% ethanol and 40% PEG400; pH 7.0) or 10 mg/kg VBP15 (10% ethanol, 10% DMSO, and 30% PEG400; pH 7.0). Oral administration (PO) was conducted by feeding CD1 mice ($n = 3$/drug) an aqueous emulsion of either VBP6 or VBP15 in 30% Labrafil. Blood samples were taken at 0-, 5-, 15-, 30 min and 1-, 2-, 4-, 8-, and 24 h.

2.6.3. Dog and rat

Sprague–Dawley rats ($n = 3$) were intravenously injected with a solution of 10 mg/kg VBP15 (10% ethanol, 10% DMSO and 30% PEG400; pH 7.0). Beagle dogs ($n = 3$) were intravenously injected with a solution of 10 mg/kg VBP15 (8% ethanol, 8% DMSO, 50% PEG400 and 34% HP-β-CD (20%W/V in water). Oral administration for SD rats ($n = 3$) and beagles ($n = 3$) was conducted by feeding an emulsion of VBP15 in 30% Labrafil. Blood samples were taken at 0-5-, 15-, 30 min and 1-, 2-, 4-, 8-, and 24 h.

2.7. Metabolic stability

Stability studies were conducted by Pharmaron using liver microsomes from human, monkey, dog, rat and mice. Either VBP compound or positive control (Verapamil) was added to microsomal solutions at a final concentration of 2 μM VBP compound, 0.5 mg/mL microsomes, 5 mM $MgCl_2$ and 5 mM PBS. The reaction was started with the addition NADPH solution at a final concentration of 1 mM and carried out at 37 °C. H_2O was used instead of NADPH solutions in the negative control. Aliquots were taken from the reaction solution at 0 and 60 min. The reaction was stopped by the addition of 3 volume of cold methanol. Aliquots of the supernatant were used for LC/MS/MS analysis for metabolite analysis and identification was performed as described above.

2.8. CYP induction

CYP1A2 and CYP3A4 induction studies were conducted by Pharmaron. Cryopreserved human liver microsomes from 3 donors (separate incubations) were obtained commercially from CellzDirect (Invitrogen). Hepatocytes were cultured on a collagen substratum for three days prior to study initiation according to manufacturer's instructions. Hepatocyte cultures were treated daily with fresh media containing 1-, 10-, and 100 μM VBP15, vehicle (negative control) or appropriate positive control (rifampin for CYP3A4 and omeprazole for CYP1A2). 24-h after the final treatment, CYP3A4 and CYP1A2 activity was determined using the FDA recommended probe substrates testosterone and phenacetin respectively. Assay analysis was conducted via LC/MS/MS. The percentage inductions relative to positive control were calculated. A greater than 40% of positive control in any one of the three donors for a CYP was considered a potential inducer of that CYP.

3. Results

3.1. In vitro screening of VBP compounds for NF-κB transrepression and GR nuclear translocation

We screened the 20 compounds for their ability to inhibit NF-κB using a luciferase reporter construct stably transfected into C_2C_{12} myogenic cells.[18] Both undifferentiated myoblasts, and differentiated, multi-nucleated myotubes were studied. VBP compounds were able to inhibit TNF-alpha induced NF-κB to varying degrees resulting in reduced percent NF-κB activity. Figure 2 displays the top 5 NF-κB inhibitors in myoblasts and myotubes. These results indicate that several of the VBP compounds are able to inhibit NF-κB at potency similar to prednisolone in vitro in muscle cells.

Cell viability was assayed on C_2C_{12} cells grown and treated concurrently to those measuring NF-κB activity. We discovered that a C16-methyl group showed strong inhibition of NF-κB and the lowest cell toxicity, while larger alkyl groups (ethyl and butyl) either decreased activity or enhanced toxicity. We now favor a Δ1,4 in the A ring because of improved metabolic stability, a Δ9,11 in the C ring, a methyl at C16, and either hydroxyl or hydrogen at C17.

Compounds were tested for potency in causing translocation of the GR from the cytoplasm to nucleus using a reporter assay in both myoblasts and myotubes.[18] EC_{50} for GR Translocation [Log M] was then plotted against the EC_{50} for NF-κB inhibition (Figure 3). VBP6, VBP7 and VBP15 clustered with prednisone and dexamethasone, showing high activities for both assays. VBP6 and VBP15 are both 21-hydroxyls with VBP7 being the acetate (prodrug) of VBP6.

3.2. VBP compounds lack transactivation activity

To determine if VBP compounds dissociate transrepression from transactivation, we screened the top NF-κB inhibiting compounds for their ability to induce GRE-dependent gene transcription using a GRE-luciferase reporter construct as we have previously

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

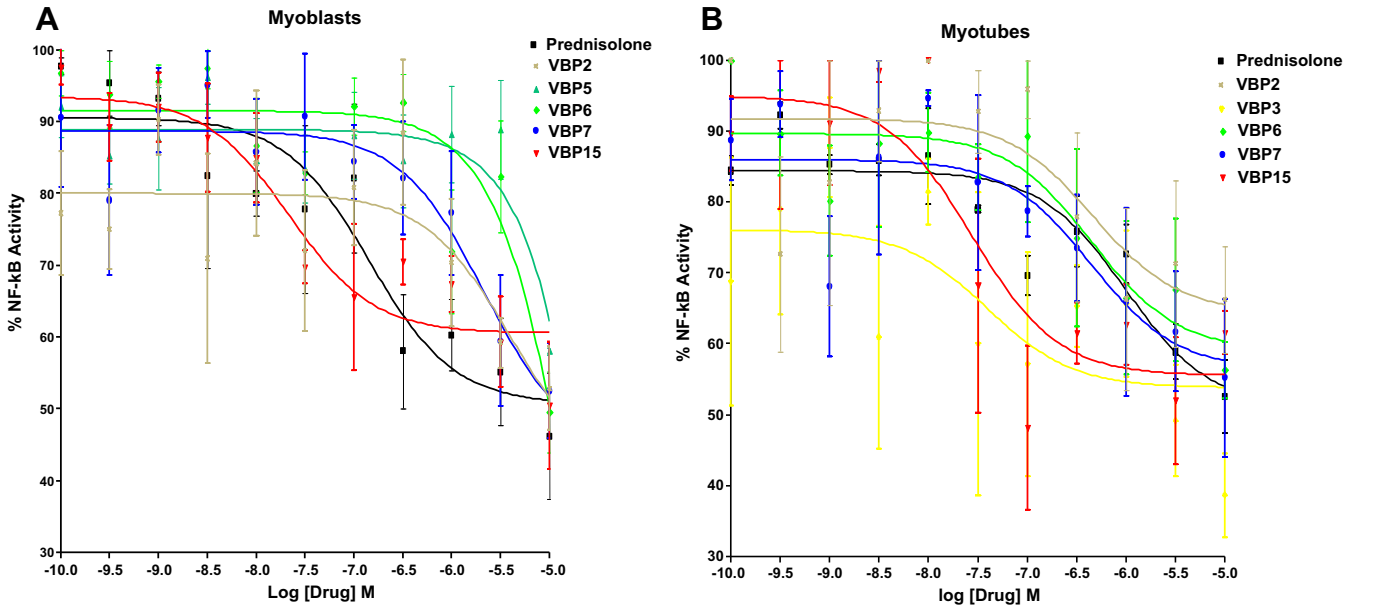


Figure 2. NF-κB inhibition in myogenic cells. (A) Dose response of the top NF-κB inhibiting VBP compounds in C_2C_{12} myoblasts. (B) Dose response of the top NF-κB inhibiting VBP compounds in C_2C_{12} differentiated myotubes. Standard deviations are shown.

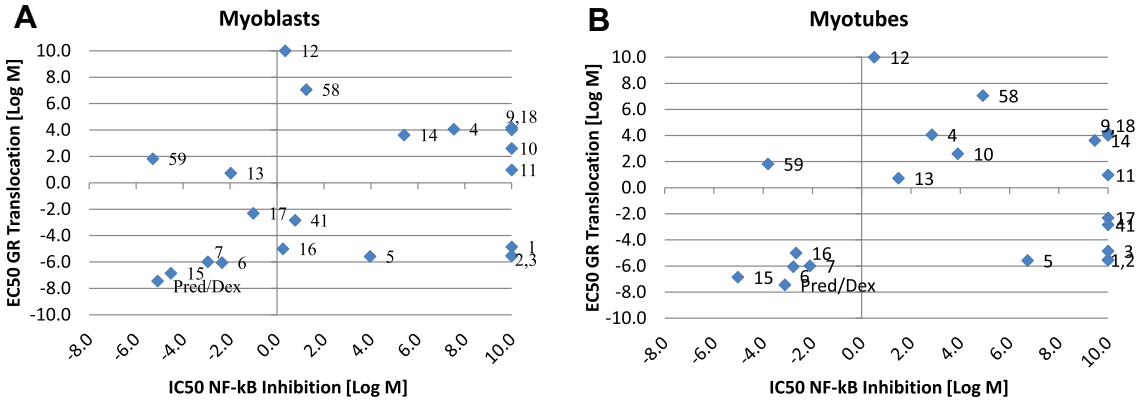


Figure 3. Correlation of NF-κB and GR nuclear translocation potency in (A) myoblasts and (B) myotubes. EC_{50} values for NF-kB inhibition activity (*X* axis) and GR nuclear translocation activity (*Y* axis) were plotted for all VBP series compounds, relative to prednisone and dexamethasone.

described.[18] Results show that there was a significant dose-dependent response in prednisolone treated cells (p <0.0001) but there was no increase in luciferase transcription at even the highest doses tested for any of the VBP compounds (Fig. 4). These data suggest we are achieving our desired SAR goals of dissociating the gene transcriptional (classic glucocorticoid) properties from the anti-inflammatory (NF-κB) properties.

3.3. Pharmacokinetics in CD1 mice following intravenous and oral administration

The two top candidates from the in vitro assays (VBP6 and VBP15) were compared for bioavailability through PK studies. The relatively poor solubility of VBP15 required formulation in 8% DMSO + 8% Ethanol + 50% PEG400 + 34% HP-β-CD (20% W/V).

PK results for VBP6 indicate that it had a relatively high clearance of 48.2 ml/min/kg, which was about 50% of the mouse blood hepatic flow. The terminal half-life was 0.67 h. Volume of distribution (V_{ss}) was 0.87 L/kg, indicating that it did not have high tissue accumulation. In the PO arm, C_{max} = 10203 ng/ml and percentage bioavailability (F%) was 128% (Table 2, Fig. 5). The F% was calculated by comparing the dose-corrected area under curve (AUC) non-intravenous divided by AUC intravenous. IV AUC was considered 100%. We had different formulations for the oral versus IV which improved solubility, and hence a larger AUC, for the oral formulation compared to the IV formulation leading to F% >100%.

PK results for VBP15 indicate that it had low-medium clearance 18.8 ml/min/kg. The terminal half-life was 0.35 h. Volume of distribution was 0.75 L/kg indicating that it also did not have high tissue accumulation. In the PO arm, C_{max} = 6787 ng/ml at 2 h, and bioavailability (F%) was 74.5% (Table 2; Fig. 5).

3.4. Metabolic stability across multiple species

The in vitro metabolic stability of VBP6 and VBP15 was studied in different species of liver microsomes. Results show ⩾80% remaining at 1 h of VBP6 and VBP15 in human, dog, and monkey microsomes (Fig. 6). VBP6 had poor stability in mouse liver microsomes (34%) compared to VBP15 (88%). Both compounds showed

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

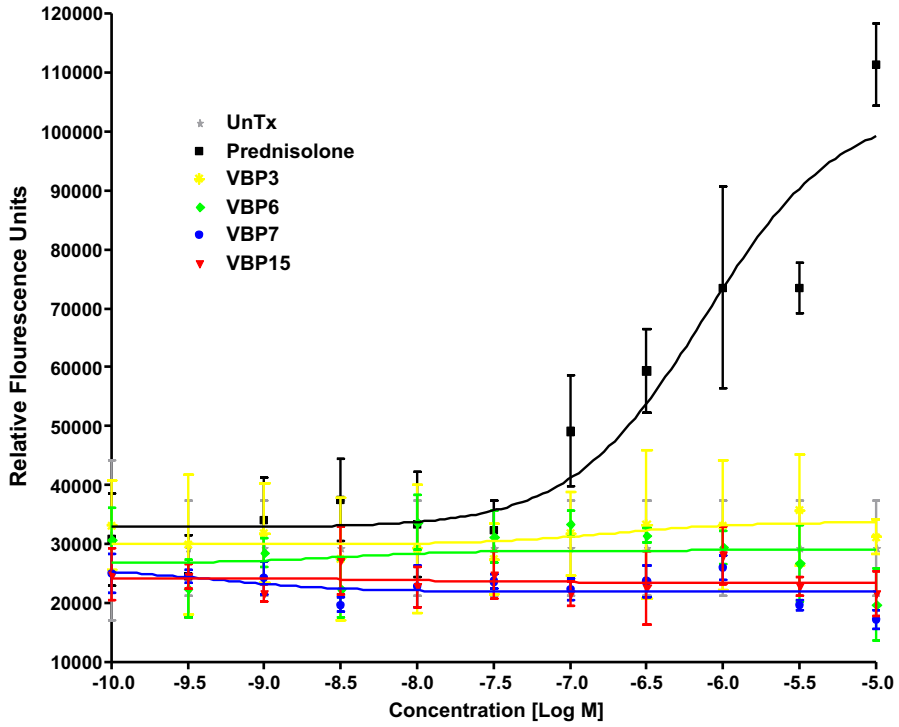


Figure 4. VBP compounds do not induce GRE-dependent gene transcription. A reporter construct with luciferase driven by glucocorticoid response elements (GREs) was studied, as previously described.[18] Prednisone induced luciferase expression, while none of the VBP series did. Standard deviations are shown.

Table 2
PK analysis of VBP6 and VBP15 following IV and PO administration in CD1 mice

Drug	Route	Dose (mg/kg)	Formulation	Cl_{obs} (mL/min/kg)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h ng/mL)	AUC_{inf} (h ng/mL)	MRT (h)	AUC/D (h ng/mL)	Vss_{obs} (L/kg)	*F* (%)
VBP6	IV	10	10% EtOH + 40% PEG400	48.2	0.667	0.0833	8.637	3.447	3.457	0.289	345	0.874	NA
	PO	50	30% Labrafil	NA	0.659	1.00	10.203	22.023	22.031	NA	440	NA	128
VBP15	IV	10	10% EtOH 10% DMSO 40% PEG400	18.8	0.354	0.0833	11.167	8.838	8.842	0.667	884	0.757	NA
	PO	50	30% Labrafil	NA	0.678	2.00	6.787	32.912	32.932	NA	658	NA	74.5

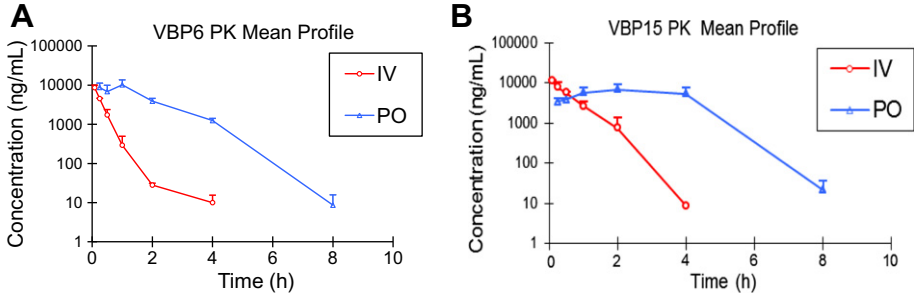


Figure 5. PK mean profiles for (A) VBP6 and (B) VBP15 in mice. Error bars indicate standard deviation.

poor stability in rat, presumably due to the high first pass clearance traditionally seen with glucocorticoids in rats.[19]

3.5. Lead compound selection

Potential lead compounds were narrowed to VBP6 and VBP15 based on superior activities (high NF-κB inhibition, low cytotoxicity, high GR binding and GR translocation). PK data showed that VBP15 had a longer T_{max} and lower C_{max} across species, leading to a greater and preferred AUC compared to VBP6. Thus, VBP15 was selected as the lead compound for all further studies.

3.6. Absorption, distribution, metabolism and excretion (ADME) and pharmacokinetics of VBP15

We performed a series of ADME and pharmacokinetic studies to characterize our lead compound. Table 3 summarizes the results of these ADME experiments. PK results for VBP15 in rats indicate that

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

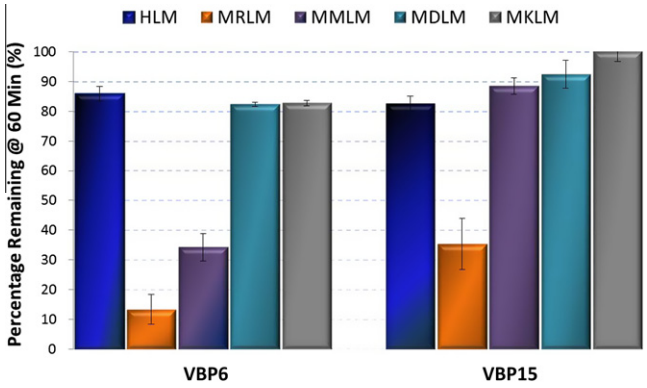


Figure 6. Metabolic stability of VBP6 and VBP15 in liver microsomes across multiple species. VBP15 shows better stability in rat and mouse microsomes compared to VBP6. HLM = human liver microsomes, MRLM = male rat liver microsomes, MMLM = male mouse liver microsomes, MDLM = male dog liver microsomes, MKLM = male monkey liver microsomes.

it had a clearance of 20.2 mL/min/kg. The terminal half-life was 0.58 h. Volume of distribution (V_{ss}) was 0.77 L/kg, indicating that it did not have high tissue accumulation. In the PO arm, C_{max} = 2543 ng/mL and percentage bioavailability (*F*%) was 47.8% (Table 4).

VBP15 in beagle dogs had medium-high clearance of 24.7 mL/min/kg. The terminal half-life was 5.42 h. Volume of distribution was 1.93 L/kg indicating that it had certain tissue distribution. In the PO arm, C_{max} = 814 ng/mL, and bioavailability (*F*%) was 53.2% (Table 4).

3.7. Metabolite identification

Human, monkey, dog, rat and mouse hepatocytes were incubated at a final concentration of 10 μM VBP15 for 2 and 4 h. Four metabolites were identified in the human and monkey hepatocytes, 3 were identified in the dog (peak 4 is the major metabolite), 5 metabolites were identified in rat (peak 3 is the major metabolite) and 2 in the mouse (Table 5).

3.8. CYP induction

There was no induction of CYP1A2 by VBP15 seen across the three donors therefore VBP15 is not considered an inducer of CYP1A2. VBP15 moderately induced CYP3A4 across the three donors (24–42%). This indicates that VBP15 is a potential inducer of CYP3A4, similar to other steroidal compounds.

4. Discussion

We have recently shown that an existing Δ9,11 steroidal compound (anecortave acetate) was able to bind and translocate the glucocorticoid receptor (GR) and inhibit anti-inflammatory pathways (NF-κB).[18] Based on these data we synthesized 20 new compounds not previously known to the literature, with the goal of carrying out a lead optimization program using the Δ9,11 backbone, in vitro screening assays included potent inhibition of NF-κB, potent translocation of the GR to the nucleus, loss of GRE-mediated transcriptional activity, and low cytotoxicity (Fig. 1, Table 1). We found that a C16-methyl group was important for NF-κB inhibitory activity, while larger alkyl groups (ethyl and butyl) either decreased activity or enhanced cytotoxicity of myogenic cells. Other optimizations of chemistry included a Δ1,4 in the A ring (improved metabolic stability), a Δ9,11 in the C ring, a methyl at C16, and either hydroxyl or hydrogen at C17. VBP15 was selected as the lead compound based on these findings (Fig. 2). VBP15 also showed desirable ADME and PK properties. Like many steroids, VBP15 shows oral absorption in a target range acceptable for oral delivery. The drug can be formulated for PK and other studies in vehicles that are typical for related steroids. PK and metabolite work suggest that the primate and mouse will probably be the preferred species for PK.

Synthetic glucocorticoids are among the most commonly prescribed drugs due to their potent anti-inflammatory properties, and remain standard of care in many conditions such as arthritis, dermatitis, asthma, muscular dystrophy, and auto-immune disorders.[20–22] However, glucocorticoids have many off-target effects that together contribute to a relatively broad side effect profile. These include but are not limited to disruption of glucose metabolism, immune suppression, adrenal suppression, thymocyte apop-

Table 3
ADME properties of VBP15

In vitro properties	Units	Value & class	Target range
Solubility (pH, media)	(μM)	187.18 μM	>60
Stability—microsomes	$t_{1/2}$ (min)	Human = 82% Rat = 35.4% Mouse = 88.8% Dog = 92.5% Monkey = 100%	>30
Stability—plasma (species)	% Remaining at 1 h	88.06%	>80%
CYP450 Inhibition (2C9,2D6,3A4)	% Inhibition at 10 mM	1.9%, 7.9%, 15.3%	<40%
	IC_{50} (mM)	>50 μM for all	>10
Permeability—Caco-2	P_{app} (a–b, 10^{-6} cm/s)	11.2	>10
	Efflux ratio	0.61	<2
hERG— (method)	IC_{50} (mM)	20 μM	>10
Free C_{max}—plasma	Total C_{max} (mM) *$F_{u,\ plasma}$	0.12	
Ames test	Positive/negative	Negative	Negative
Micronucleus test	Positive/negative	Negative	Negative
Blood Brain Barrier (rats)[a]	Ratio (brain/plasma) (h)	0.25 = 0.648 0.5 = 0.620 1.0 = 0.614 2.0 = 0.593 4.0 = 0.583 6.0 = 0.581 8.0 = 0.433	

[a] The brain concentration was not corrected for vascular content.

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

Table 4
PK properties of VBP15 across species

PK properties	Units	Mouse 10 mpk IV 50 mpk PO	Rat 10 mpk IV 50 mpk PO	Dog 10 mpk IV 30 mpk PO	Target range
$t_{1/2}$	h	0.354 (IV) 0.678 (PO)	0.58 (IV) 2.29 (PO)	5.42 (IV) 2.25 (PO)	>3
$AUC_{0-\infty,\ total,\ unbound}$	h ng/mL	8842 (IV) 32932 (PO)	8339 (IV) 19937 (PO)	6791 (IV) 10844 (PO)	>500 (PO)
CL	mL/min/kg	18.8 (IV)	20.2 (IV)	24.7 (IV)	<25% HBF
$C_{max,\ total,\ unbound}$	ng/mL (nM)	11167 (IV) 6787 (PO)	2543 (PO)	814 (PO)	
T_{max}	h	0.083 (IV) 2.0 (PO)	4.00 (PO)	6.00 (PO)	
V_{ss}	L/kg	0.757 (IV)	0.770 (IV)	1.93 (IV)	
F	%	74.5	47.8	53.2	>20%

Table 5
VBP15 metabolites identified across species

Peak	Rt	Expected	Mass	Biotransformation	Metabolites in hepatocytes				
no.	(min)	m/z	shift		Human	Monkey	Dog	Rat	Mouse
1	5.52	371.2	16	Oxidation	+	+	+	+	+
2	6.37	373.2	18	Oxidation + hydrogenation	+	+	–	+	–
3	7.78	371.2	16	Oxidation	–	–	–	+++	–
4	9.69	369.2	14	Methylation	+	+	+++	+	+
5	10.42	355.2	0	Parent drug	+++	+++	+	+	+++
6	12.86	357.2	2	Hydrogenation	+	+	+	+	–

tosis, osteoporosis, erythroblast proliferation, elevation of intraocular pressure, cataract, and mood changes. Many of these side effect profiles are associated with GRE-mediated transcriptional activities of steroids (classic glucocorticoid activity). We have shown that VBP15 lacks GRE-mediated transcriptional activity (Fig. 2), although it inhibits NF-κB and causes translocation of the GR to the nucleus. We should note that classic glucocorticoids result in nuclear translocation as ligand/GR dimmers able to bind GRE elements (transactivation properties), as well as ligand/GR complexes with NF-κB transcriptional complexes that target NF-κB promoter elements (transrepression properties). VBP15 appears to disassociate these two subactivities, with GR translocation likely due to NF-κB transcriptional complexes. Thus, we expect that preclinical studies will show superior side effect profiles of VBP15 compared to other steroid-based anti-inflammatories, as we have previously shown for anecortave acetate.[18]

The extensive effect profiles of pharmacological glucocorticoids often limit prescription despite proven efficacy, particularly in children. For example, in Duchenne muscular dystrophy, daily prednisone is considered standard of care in some countries, but the efficacy if offset by increased bone fragility, mood changes, weight gain, and muscle catabolic pathways.[23–26] Thus, there is non-adoption of this standard in some countries, and relatively poor adherence even in those countries adopting daily glucocorticoids.[27] The VBP15 described here is being developed for Duchenne muscular dystrophy as the initial indication, in collaboration with the Muscular Dystrophy Association Venture Philanthropy, and National Institutes of Health Therapeutics for Rare and Neglected Disease program. It is our hope that VBP15 not only provides a superior side effect profile relative to prednisone, but will also improve efficacy by opening the therapeutic window (allowing increased dosing relative to prednisone).

Acknowledgments

Supported in part by Grants from the Congressionally Directed Medical Research Program of the US Department of Defense (W81XWH-09-1-0218; W81XWH-11-1-0754), Muscular Dystrophy Association Venture Philanthropy (Phase 1 and Phase 2), Foundation to Eradicate Duchenne, NIH Wellstone Muscular Dystrophy Research Center (1U54HD053177-01A1), and NIH Therapeutics for Rare and Neglected Disease (TRND) program.

References and notes

1. Hall, E. D.; Braughler, J. M.; McCall, J. M. *Prog. Clin. Biol. Res.* **1990**, *361*, 351–362.
2. Bracken, M. B.; Shepard, M. J.; Holford, T. R.; Leo-Summers, L.; Aldrich, E. F.; Fazl, M.; Fehlings, M.; Herr, D. L.; Hitchon, P. W.; Marshall, L. F.; Nockels, R. P.; Pascale, V.; Perot, P. L., Jr.; Piepmeier, J.; Sonntag, V. K.; Wagner, F.; Wilberger, J. E.; Winn, H. R.; Young, W. *JAMA* **1997**, *277*, 1597–1604.
3. Bracken, M. B.; Shepard, M. J.; Holford, T. R.; Leo-Summers, L.; Aldrich, E. F.; Fazl, M.; Fehlings, M. G.; Herr, D. L.; Hitchon, P. W.; Marshall, L. F.; Nockels, R. P.; Pascale, V.; Perot, P. L., Jr.; Piepmeier, J.; Sonntag, V. K.; Wagner, F.; Wilberger, J. E.; Winn, H. R.; Young, W. *J. Neurosurg.* **1998**, *89*, 699–706.
4. Lanzino, G.; Kassell, N. F.; Dorsch, N. W.; Pasqualin, A.; Brandt, L.; Schmiedek, P.; Truskowski, L. L.; Alves, W. M. *J. Neurosurg.* **1999**, *90*, 1011–1017.
5. Lanzino, G.; Kassell, N. F. *J. Neurosurg.* **1999**, *90*, 1018–1024.
6. van der Worp, H. B.; Kappelle, L. J.; Algra, A.; Bär, P. R.; Orgogozo, J. M.; Ringelstein, E. B.; Bath, P. M.; van Gijn, J. *Neurology* **2002**, *58*, 133.
7. Kavanagh, R. J.; Kam, P. C. *Br. J. Anaesth.* **2001**, *86*, 110–119.
8. Wendler, A.; Wehling, M. *Steroids* **2010**, *75*, 619–623.
9. Flessas, I. I.; Papalois, A. E.; Toutouzas, K.; Zagouri, F.; Zografos, G. C. *J. Surg. Res.* **2011**, *166*, 265–274.
10. McNatt, L. G.; Weimer, L.; Yanni, J., et al *J. Ocul. Pharmacol. Ther.* **1999**, *15*, 413–423.
11. Penn, J. S.; Rajaratnam, V.; Collier, R. J.; Clark, A. F. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 283.
12. D'Amico, D. J.; Goldberg, M. F.; Hudson, H.; Jerdan, J. A.; Krueger, D. S.; Luna, S. P.; Robertson, S. M.; Russell, S.; Singerman, L.; Slakter, J. S.; Yannuzzi, L.; Zilliox, P. *Ophthalmology* **2003**, *110*, 2372. discussion 2384–5.
13. Slakter, J. S.; Bochow, T. W.; D'Amico, D. J.; Marks, B.; Jerdan, J.; Sullivan, E. K.; Robertson, S. M.; Slakter, J. S.; Sullins, G.; Zilliox, P. *Ophthalmology* **2006**, *113*, 3–13.
14. Stalmans, I.; Callanan, D. G.; Dirks, M. S.; Moster, M. R.; Robin, A. L.; Van Calster, J.; Scheib, S. A.; Dickerson, J. E., Jr.; Landry, T. A.; Bergamini, M. V. *J. Ocul. Pharmacol. Ther.* **2012**, *28*, 559–565.
15. Robin, A. L.; Suan, E. P.; Sjaarda, R. N.; Callanan, D. G.; Defaller, J. *Arch. Ophthalmol.* **2009**, *127*, 173–178.
16. Robin, A. L.; Clark, A. F.; Covert, D. W.; Krueger, S.; Bergamini, M. V.; Landry, T. A., ; Dickerson, J. E., Jr.; Scheib, S. A.; Realini, T.; Defaller, J. M.; Cagle, G. D. *Am. J. Ophthalmol.* **2009**, *147*, 45.
17. Baudy, A. R.; Reeves, E. K.; Damsker, J. M.; Heier, C.; Garvin, L. M.; Dillingham, B. C.; McCall, J.; Rayavarapu, S.; Wang, Z.; Vandermeulen, J. H.; Sali, A.; Jahnke, V.; Duguez, S.; DuBois, D.; Rose, M. C.; Nagaraju, K.; Hoffman, E. P. *J. Pharmacol. Exp. Ther.* **2012**, *343*, 225–232.
18. Baudy, A. R.; Saxena, N.; Gordish, H.; Hoffman, E. P.; Nagaraju, K. *Int. Immunopharmacol.* **2009**, *9*, 1209–1214.
19. Haughey, D. B.; Jusko, W. J. *J. Pharm. Sci.* **1992**, *81*, 117–121.

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

20. Bijlsma, J. W. *Rheumatology (Oxford)* **2012**, *51*, iv9.
21. Butterfield, J. H.; Weiler, C. R. *Semin. Hematol.* **2012**, *49*, 182–191.
22. LeHoang, P. *Dev. Ophthalmol.* **2012**, *51*, 7–28.
23. Sandri, M.; Sandri, C.; Gilbert, A.; Skurk, C.; Calabria, E.; Picard, A.; Walsh, K.; Schiaffino, S.; Lecker, S. H.; Goldberg, A. L. *Cell* **2004**, *117*, 399–412.
24. Zheng, B.; Ohkawa, S.; Li, H.; Roberts-Wilson, T. K.; Price, S. R. *FASEB J.* **2010**, *24*, 2660–2669.
25. Gokulakrishnan, G.; Estrada, I. J.; Sosa, H. A.; Fiorotto, M. L. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2012**, *302*, R1143–R1152.
26. Reeves, E. K.; Rayavarapu, S.; Damsker, J. M.; Nagaraju, K. *Endocr. Metab. Immune Disord. Drug Targets* **2012**, *12*, 95–103.
27. Hoffman, E. P.; Reeves, E.; Damsker, J.; Nagaraju, K.; McCall, J. M.; Connor, E. M.; Bushby, K. *Phys Med Rehabil Clin N Am.* **2012**, *23*, 821–828.

Please cite this article in press as: Reeves, E. K.; et al. *Bioorg. Med. Chem.* (2013), http://dx.doi.org/10.1016/j.bmc.2013.02.009

Bioorganic & Medicinal Chemistry 21 (2013) 2241–2249

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

VBP15: Preclinical characterization of a novel anti-inflammatory delta 9,11 steroid

Erica K. M. Reeves [a,*], Eric P. Hoffman [a,b,d], Kanneboyina Nagaraju [a,b,d], Jesse M. Damsker [a], John M. McCall [a,c]

[a] ReveraGen BioPharma, Inc., 9700 Great Seneca Hwy Rockville, MD 20910, United States
[b] Center for Genetic Medicine Research, Children's National Medical Center, 111 Michigan Ave. NW, Washington, DC 20010, United States
[c] PharMac, LLC, P.O. Box 2253, Boca Grande, FL 33921 United States
[d] Department of Integrative Systems Biology, George Washington University School of Medicine and Health Sciences, Washington, DC 20052, United States

ARTICLE INFO

Article history:
Received 24 December 2012
Revised 3 February 2013
Accepted 11 February 2013
Available online 18 February 2013

Keywords:
Glucocorticoids
VBP15
Steroids
Anti-inflammatories
Neuromuscular disease

ABSTRACT

Δ9,11 modifications of glucocorticoids (21-aminosteroids) have been developed as drugs for protection against cell damage (lipid peroxidation; lazaroids) and inhibition of neovascularization (anecortave). Part of the rationale for developing these compounds has been the loss of glucocorticoid receptor binding due to the Δ9,11 modification, thus avoiding many immunosuppressive activities and deleterious side effect profiles associated with binding to glucocorticoid and mineralocorticoid receptors. We recently demonstrated that anecortave acetate and its 21-hydroxy analog (VBP1) do, in fact, show glucocorticoid and mineralocorticoid receptor binding activities, with potent translocation of the glucocorticoid receptor to the cell nucleus. We concluded that Δ9,11 steroids showed novel anti-inflammatory properties, retaining NF-κB inhibition, but losing deleterious glucocorticoid side effect profiles. Evidence for this was developed in pre-clinical trials of chronic muscle inflammation. Here, we describe a drug development program aimed at optimizing the Δ9,11 chemistry. Twenty Δ9,11 derivatives were tested in in vitro screens for NF-κB inhibition and GR translocation to the nucleus, and low cell toxicity. VBP15 was selected as the lead compound due to potent NF-κB inhibition and GR translocation similar to prednisone and dexamethasone, lack of transactivation properties, and good bioavailability. Phamacokinetics were similar to traditional glucocorticoid drugs with terminal half-life of 0.35 h (mice), 0.58 h (rats), 5.42 h (dogs), and bioavailability of 74.5% (mice), and 53.2% (dogs). Metabolic stability showed ⩾80% remaining at 1 h of VBP6 and VBP15 in human, dog, and monkey liver microsomes. Solubility, permeability and plasma protein binding were within acceptable limits. VBP15 moderately induced CYP3A4 across the three human hepatocyte donors (24–42%), similar to other steroids. VBP15 is currently under development for treatment of Duchenne muscular dystrophy.

1. Introduction

Δ9,11 Steroids (21-aminosteroids) have been designed and developed to stably incorporate into cell membranes and inhibit lipid peroxidation without glucocorticoid or mineralocorticoid activities, thus avoiding side effects associated with traditional corticosteroids.[1] A few Δ9,11 steroids brought to clinical trials were Lazaroids and Anecortave. Lazaroids have extensive hydrophilic head groups designed to optimize stability in membranes and lipid peroxidation inhibitory activities (Fig. 1). Randomized controlled trials of tirilazad mesylate in acute spinal cord injury,[2,3] and in stroke[4–6] showed good safety profiles, but failed to show clear evidence of efficacy. Pre-clinical work in additional indications continues, including multiple wound states and lung disease.[7–9]

Anecortave was developed for inhibition of neo-vascularization in age-related macular degeneration as a molecule that lacked immunomodulatory glucocorticoid activity.[10,11] The mechanism of action is thought to be through inhibition of proliferation of vascular endothelial cells, inhibition of uPA and matrix metalloproteinase 3, and stimulation of plasminogen activator inhibitor-1.[11] Controlled clinical trials of posterior juxtascleral depot (delivered at 6 month intervals) showed no safety issues, but marginal clinical benefit.[12,13] More recently, blinded controlled trials of anecortave in steroid-induced elevated intraocular pressure (IOP) have been carried out, with similar results of good safety profile.[14] The best dose of anecortave acetate showed a 31% reduction in IOP and this benefit lasted a mean duration of 56 days from a single administration.[15,16]

Previous drug development programs pursuing lazaroids and anecortave as prototype Δ9,11 glucocorticoids (21-aminosteroids) have assumed that the Δ9,11 modification inhibited drug binding to the glucocorticoid receptor (GR) and mineralocorticoid receptor

* Corresponding author. Tel.: +1 (202) 476 1236; fax: +1 (240) 453 6208.
E-mail address: erica.reeves@reveragen.com (E.K.M. Reeves).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.02.009

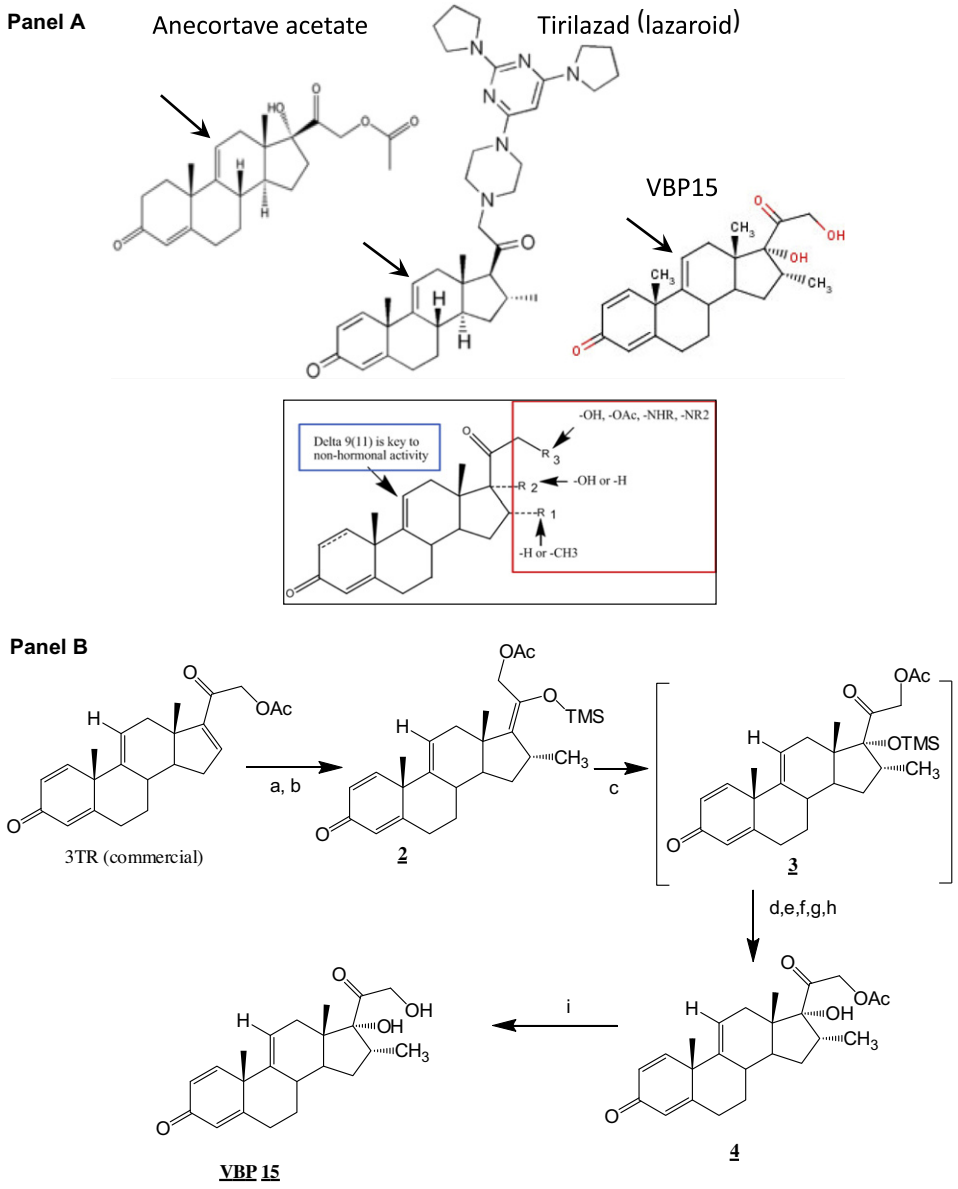


Figure 1. Structures and synthesis of Δ9,11 compounds. Panel A: Shown are two previously developed Δ9,11 steroids (Anecortave, Tirilizad), and the lead compound identified in the current study (VBP15). The Δ9,11 bond distinguishing these drugs from other steroidal compounds is shown by the black arrows. The lower panel shows the chemical modifications to the chemical backbone. The R1, R2, and R3 residues shown are those that were chemically modified with alternative chemical structures (see red box). Panel B: The synthesis of VBP15 that is pictured is typical for the synthesis of analogs in this series. (a) TMS imidazole, MeMgCl, THF; (b) $CuAc_2$, H_2O, DMPU, MeMgCl, THF; (c) peracetic acid, toluene, −10 °C; (d) $NaHSO_3$, TFA; (e) EtOAc, heptanes; (f) acetonitrile trituaration; (g) HBr, HC_2Cl_2, 40 °C; (h) MeOH crystallization; (i) K_2CO_3, MeOH, followed by HCL and crystallization from $MeOH/H_2O$.

(MR). This assumption was based largely on the lack of efficacy of the drugs in inhibiting LPS-induced inflammation in pre-clinical models—traditional glucocorticoids are impressively effective at blocking lipopolysaccharide (LPS)-induced acute inflammation.[10] However, we recently showed that both anecortave acetate and its 21-hydroxy analog (VBP1) showed relatively high affinity binding to both the GR and MR.[17] The Δ9,11 drugs induced GR translocation from the cell cytoplasm to cell nucleus, shown by both reporter and immunostaining assays, again suggesting effective ligand/receptor interactions. Nuclear translocation of steroid hormone receptors is a hallmark of classic glucocorticoid transcriptional activity, and is necessary for downstream activation of gene promoters via binding of ligand/receptor complexes to glucocorticoid response elements (GREs). However, neither Δ9,11 drug showed significant GRE-mediated transcriptional activities, either by mRNA microarray, targeted RT-PCR, or luciferase reporter constructs.[17] On the other hand, both drugs retained anti-inflammatory activity via inhibition of NF-κB pathways, and showed efficacy in inhibiting inflammation in vivo in two mouse models of chronic inflammation in muscle (dystrophin-deficient *mdx* and dysferlin-deficient *SJL* mice).[17] The discrepancy between previous findings of lack of anti-inflammatory activity, and our findings of retention of anti-inflammatory activity may be explained by the assays utilized. For example, McNatt et al. studied an acute model of LPS-induced IL-1 induction, where anecortave failed to induce IL-1, whereas glucocorticoids were effective in induction.[10] Our studies focused on in vitro assays of NF-κB inhibition and models of chronic immunity not utilizing LPS.[17]

Here, we queried the chemical space around the Δ9,11 steroid backbone, optimizing for anti-inflammatory properties (NF-κB

inhibition and GR nuclear translocation). The lead compound, VBP15, shows excellent drug properties, and is in development for Duchenne muscular dystrophy as the initial indication.

2. Materials and methods

2.1. Chemistry

All compounds were synthesized by Bridge Organics Co. (Kalamazoo, Michigan). Systematic (IUPAC) names: Anecortave ([2-[(8*R*,10*S*,13*S*,14*R*,17*R*)-17-hydroxy-10,13-dimethyl-3-oxo-2,6,7,8,12,14,15,16-octahydro-1*H*-cyclopenta[*a*]phenanthren-17-yl]-2-oxo-ethyl] acetate). Tirilazad ((16α)-21-[4-(2,6-dipyrrolidin-1-yl-pyrimidin-4-yl)piperazin-1-yl]-16-methylpregna-1,4,9(11)-triene-3,20-dione). Additional chemical structures studied are described in Table 1.

Traditional glucocorticoids have an 11-beta-hydroxy or an 11-keto group whereas we used a double bond between carbons 9 and 11, leading to a Δ9,11 C ring as the backbone, then developed a series of steroids based on this scaffold (Fig. 1, Panel A; Table 1). These scaffolds are not oxidatively metabolized to 11-beta hydroxy compounds, and should have less off-target effects related to active metabolites. We varied the D ring of the steroid, probing the effect of different lipophilic groups at C16 and either hydroxy or hydrogen at C17. Twenty compounds were synthesized in the VBP series of Δ9,11 compounds varying in structure at the three positions (R1, R2, R3) (Fig. 1; Table 1).

The synthesis of the Δ9,11 steroid begins with commercially available steroid 3TR (Fig. 1; Panel B). Copper catalyzed Michael addition of MeMgCl with TMSCl through a 1,4 addition introduced a 16-alpha methyl group to give silyl enol ether **2** with 93% purity by hplc/ms and in nearly quantitative yield. The product was isolated as a toluene slurry which was oxidized with 32% by weight peracetic acid in acetic acid to yield intermediate **3** which was not isolated but was converted to compound **4** as a powder that precipitated from EtOAc/heptane trituration in 60% yield from 3TR and with 86% purity. In order to remove a small amount of the delta 9,11 epoxide, compound **4** was treated with HBr in methylene chloride. Yield from the HBr treatment after MeOH/water crystallization was 90% with 97% purity and an overall yield from 3TR of 54%. Finally, the acetate of compound **4** is removed by K_2CO_3 hydrolysis. Methanol/water crystallization of VBP15 gave a 79% yield with 98.3% purity. Overall yield from 3TR is 43%. Purity is assigned by hplc/ms. Structure was confirmed by proton and carbon NMR.

2.2. NF-κB screen

C_2C_{12} skeletal muscle cells stably transfected with a luciferase reporter construct regulated under NF-κB response element (Panomics, Fremont, CA) were used for screening NF-κB inhibitors, as previously described.[18] Myoblasts and myotubes grown in growth and differentiating medium respectively were pretreated with various concentrations (vehicle [DMSO]; 0.01–100 μg/ml) of the drugs for a 24 h duration before stimulating with tumor necrosis factor-α (TNF-α) (10 ng/ml) for another 24 h. After the completion of incubation, cells were washed once with PBS and lysed with cell lysis buffer to measure luciferase activity (Promega Corp, Madison, WI) using Centro LB 960 luminometer (Berthold technologies, GmbH & Co, Bad Wildbad, Germany) and IC_{50} values are calculated for each compound.

2.3. Cell viability

Cell viability was determine by MTT assay (3-[4,5 dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) (Sigma, St., Louis, Missouri) as per manufacturer's protocols. Percent cell viability was calculated relative to untreated cells, whereas relative luminescence units with TNF-α stimulation in the absence of drugs were considered as 100% percent. Data are represented as % inhibition relative to TNF-α induced NF-κB activation. Significance was calculated using a one-way repeated measure ANOVA to determine if there is an effect of drug concentration on cell viability.

2.4. Nuclear translocation assays

Translocation assays were performed by DiscoveRx (Fremont, CA) using GR Nuclear Translocation PathHunter cells (DiscoveRx; Fremont, CA). This assay is based on the detection of protein–protein interactions between the GR and a nuclear fusion protein containing steroid receptor co-activator peptide. The receptor is tagged with the ProLink component of enzyme fragment complementation assay system and the steroid receptor co-activator peptide (SRCP) is fused to the enzyme acceptor component. When the receptor is bound by ligand it translocates to the nucleus where it

Table 1
Chemical structures of compounds tested

VBP#	A	16	17	21	NF-κB inhibition EC_{50} (M)	Rank NF-κB	GR nuclear translocation (%)	Rank GR translocation	Sum rank
Pred/Dex					1.67E-08	1	100.00	1	2
6	Delta 1,4	CH_3	H	OH	5.64E-08	3	54.20	4	7
15	Delta 1,4	CH_3	OH	OH	6.59E-08	6	80.40	2	8
7	Delta 1,4	CH_3	H	OAc	5.41E-08	2	48.80	7	9
1	Delta 4	H	OH	OH	6.29E-08	5	49.20	6	11
16	Delta 1,4	Beta CH_3	OH	OH	6.23E-08	4	44.50	8	12
2	Delta 1,4	H	OH	OH	1.29E-07	9	57.60	3	12
3	Delta 4	H	OH	Oac	2.12E-07	10	51.50	5	15
41	Delta 1,4	Ethyl	H	OH	8.95E-08	7	27.00	11	18
17	Delta 4	CH_3	H	Dipropylamino	7.23E-07	13	37.70	9	22
11	Delta 4	H	OH	Morpholino	1.74E-06	15	28.70	10	25
5	Delta 1,4	ene	ene	OAc	9.36E-08	8	15.30	18	26
13	Delta 4	H	OH	Dipropylamino	4.35E-07	11	21.80	15	26
10	Delta 4	H	OH	Pyrrolidine	1.85E-06	16	24.70	12	28
4	Delta 1,4	ene	ene	OH	4.95E-07	12	16.70	17	29
18	Delta 4	CH_3	H	Pyrrolidine	9.88E-07	14	19.90	16	30
14	Delta 4	CH_3	H	Morpholino	5.24E-06	17	22.70	14	31
9	Delta 4	H	OH	N-Me Piperazine	5.78E-06	18	23.50	13	31
12	Delta 4	Me	H	N-Me Piperazine	>1e-5	21	14.70	19	40
59	Delta 4	*n*Butyl	H	OH	>1e-5	21	8.50	20	41
58	Delta 1,4	*n*Butyl	H	OH	>1e-5	21	6.90	21	42

recruits the SRCP and complementation occurs producing a chemiluminescent signal. A ten point agonist dose curve, ranging from 1×10^{-5} to 1.69×10^{-10} M was performed on all VBP compounds. Dexamethasone was performed in parallel as the standard reference control. Percentage activity is calculated using the following formula: % Activity = 100% × (Mean Relative Light Units [RLU] of test sample—mean RLU of vehicle control)/(mean MAX RLU control ligand—mean RLU of vehicle control)). EC_{50} is calculated for all the compounds.

2.5. Transactivation assays

HEK-293 cells stably transfected with a luciferase reporter construct regulated under glucocorticoid response element (GRE) (Panomics, Fremont, CA) were grown according to manufacturer's instructions. Cells were treated with various concentrations (0.01 to 100 μg/ml) of the drug for 6 h. Cells were washed once with PBS and lysed with cell lysis buffer to measure luciferase activity (Promega Corp, Madison, WI) using Centro LB 960 luminometer (Berthold technologies, GmbH & Co, Bad Wildbad, Germany).

2.6. Pharmacokinetics

Pharmacokinetic studies were carried out at Pharmaron (US headquarters 6 Venture, Suite 250, Irvine, CA 92618; work performed in Pharmaron-Beijing, 6 Taihe Road, BDA Beijing, 100176). Mice were purchased from Huafukang Bioscience, Beijing, China. Dogs were purchased from Marshall Inc., Beijing, China. Monkeys were purchased from Hainan, Inc., Jinggang, China and Guangxi Inc., Guidong China.

2.6.1. Analysis

All analyses were conducted on a Shimadzu liquid chromatograph separation system equipped with degasser DGU-20A3, solvent delivery unit LC-20AD, system controller CBM-20A, column oven CTO-10ASVP and CTC Analytics HTC PAL System. . Samples were loaded onto a Phenomenex Luna 5μ C18 (2) (2.0 × 50 mm) coupled with a preguard column with a mobile phase of 0.1% formic acid in acetonitrile (A) and 0.1% formic acid in water. Mass spectrometric analysis was performed using an API 4000 instrument from AB Inc (Canada) with an ESI interfaceThe data acquisition and control system were created using Analyst 1.5 software from ABI Inc.

2.6.2. Mouse

CD1 mice (n = 3/drug) were injected via tail vein with a solution of 10 mg/kg VBP6 (10% ethanol and 40% PEG400; pH 7.0) or 10 mg/kg VBP15 (10% ethanol, 10% DMSO, and 30% PEG400; pH 7.0). Oral administration (PO) was conducted by feeding CD1 mice (n = 3/drug) an aqueous emulsion of either VBP6 or VBP15 in 30% Labrafil. Blood samples were taken at 0-, 5-, 15-, 30 min and 1-, 2-, 4-, 8-, and 24 h.

2.6.3. Dog and rat

Sprague–Dawley rats (n = 3) were intravenously injected with a solution of 10 mg/kg VBP15 (10% ethanol, 10% DMSO and 30% PEG400; pH 7.0). Beagle dogs (n = 3) were intravenously injected with a solution of 10 mg/kg VBP15 (8% ethanol, 8% DMSO, 50% PEG400 and 34% HP-β-CD (20%W/V in water). Oral administration for SD rats (n = 3) and beagles (n = 3) was conducted by feeding an emulsion of VBP15 in 30% Labrafil. Blood samples were taken at 0-5-, 15-, 30 min and 1-, 2-, 4-, 8-, and 24 h.

2.7. Metabolic stability

Stability studies were conducted by Pharmaron using liver microsomes from human, monkey, dog, rat and mice. Either VBP compound or positive control (Verapamil) was added to microsomal solutions at a final concentration of 2 μM VBP compound, 0.5 mg/mL microsomes, 5 mM $MgCl_2$ and 5 mM PBS. The reaction was started with the addition NADPH solution at a final concentration of 1 mM and carried out at 37 °C. H_2O was used instead of NADPH solutions in the negative control. Aliquots were taken from the reaction solution at 0 and 60 min. The reaction was stopped by the addition of 3 volume of cold methanol. Aliquots of the supernatant were used for LC/MS/MS analysis for metabolite analysis and identification was performed as described above.

2.8. CYP induction

CYP1A2 and CYP3A4 induction studies were conducted by Pharmaron. Cryopreserved human liver microsomes from 3 donors (separate incubations) were obtained commercially from CellzDirect (Invitrogen). Hepatocytes were cultured on a collagen substratum for three days prior to study initiation according to manufacturer's instructions. Hepatocyte cultures were treated daily with fresh media containing 1-, 10-, and 100 μM VBP15, vehicle (negative control) or appropriate positive control (rifampin for CYP3A4 and omeprazole for CYP1A2). 24-h after the final treatment, CYP3A4 and CYP1A2 activity was determined using the FDA recommended probe substrates testosterone and phenacetin respectively. Assay analysis was conducted via LC/MS/MS. The percentage inductions relative to positive control were calculated. A greater than 40% of positive control in any one of the three donors for a CYP was considered a potential inducer of that CYP.

3. Results

3.1. In vitro screening of VBP compounds for NF-κB transrepression and GR nuclear translocation

We screened the 20 compounds for their ability to inhibit NF-κB using a luciferase reporter construct stably transfected into C_2C_{12} myogenic cells.[18] Both undifferentiated myoblasts, and differentiated, multi-nucleated myotubes were studied. VBP compounds were able to inhibit TNF-alpha induced NF-κB to varying degrees resulting in reduced percent NF-κB activity. Figure 2 displays the top 5 NF-κB inhibitors in myoblasts and myotubes. These results indicate that several of the VBP compounds are able to inhibit NF-κB at potency similar to prednisolone in vitro in muscle cells.

Cell viability was assayed on C_2C_{12} cells grown and treated concurrently to those measuring NF-κB activity. We discovered that a C16-methyl group showed strong inhibition of NF-κB and the lowest cell toxicity, while larger alkyl groups (ethyl and butyl) either decreased activity or enhanced toxicity. We now favor a Δ1,4 in the A ring because of improved metabolic stability, a Δ9,11 in the C ring, a methyl at C16, and either hydroxyl or hydrogen at C17.

Compounds were tested for potency in causing translocation of the GR from the cytoplasm to nucleus using a reporter assay in both myoblasts and myotubes.[18] EC_{50} for GR Translocation [Log M] was then plotted against the IC_{50} for NF-κB inhibition (Figure 3). VBP6, VBP7 and VBP15 clustered with prednisone and dexamethasone, showing high activities for both assays. VBP6 and VBP15 are both 21-hydroxyls with VBP7 being the acetate (prodrug) of VBP6.

3.2. VBP compounds lack transactivation activity

To determine if VBP compounds dissociate transrepression from transactivation, we screened the top NF-κB inhibiting compounds for their ability to induce GRE-dependent gene transcription using a GRE-luciferase reporter construct as we have previously

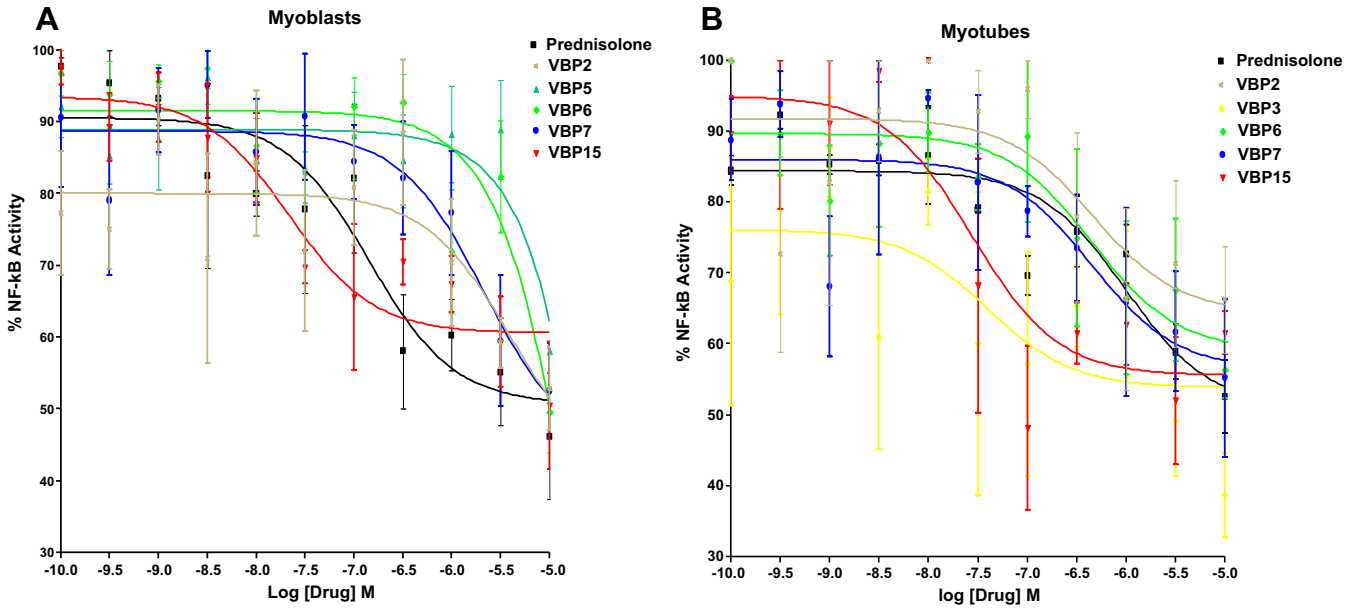


Figure 2. NF-κB inhibition in myogenic cells. (A) Dose response of the top NF-κB inhibiting VBP compounds in C_2C_{12} myoblasts. (B) Dose response of the top NF-κB inhibiting VBP compounds in C_2C_{12} differentiated myotubes. Standard deviations are shown.

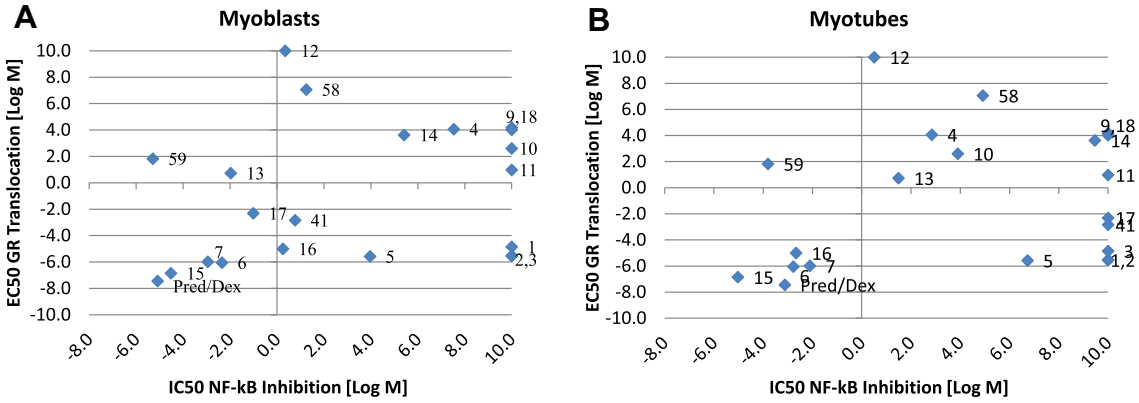


Figure 3. Correlation of NF-κB and GR nuclear translocation potency in (A) myoblasts and (B) myotubes. IC_{50} values for NF-kB inhibition activity (*X* axis) and GR nuclear translocation activity (*Y* axis) were plotted for all VBP series compounds, relative to prednisone and dexamethasone.

described.[18] Results show that there was a significant dose-dependent response in prednisolone treated cells (p <0.0001) but there was no increase in luciferase transcription at even the highest doses tested for any of the VBP compounds (Fig. 4). These data suggest we are achieving our desired SAR goals of dissociating the gene transcriptional (classic glucocorticoid) properties from the anti-inflammatory (NF-κB) properties.

3.3. Pharmacokinetics in CD1 mice following intravenous and oral administration

The two top candidates from the in vitro assays (VBP6 and VBP15) were compared for bioavailability through PK studies. The relatively poor solubility of VBP15 required formulation in 8% DMSO + 8% Ethanol + 50% PEG400 + 34% HP-β-CD (20% W/V).

PK results for VBP6 indicate that it had a relatively high clearance of 48.2 ml/min/kg, which was about 50% of the mouse blood hepatic flow. The terminal half-life was 0.67 h. Volume of distribution (V_{ss}) was 0.87 L/kg, indicating that it did not have high tissue accumulation. In the PO arm, C_{max} = 10203 ng/ml and percentage bioavailability (*F*%) was 128% (Table 2, Fig. 5). The *F*% was calculated by comparing the dose-corrected area under curve (AUC) non-intravenous divided by AUC intravenous. IV AUC was considered 100%. We had different formulations for the oral versus IV which improved solubility, and hence a larger AUC, for the oral formulation compared to the IV formulation leading to *F*% >100%.

PK results for VBP15 indicate that it had low-medium clearance 18.8 ml/min/kg. The terminal half-life was 0.35 h. Volume of distribution was 0.75 L/kg indicating that it also did not have high tissue accumulation. In the PO arm, C_{max} = 6787 ng/ml at 2 h, and bioavailability (*F*%) was 74.5% (Table 2; Fig. 5).

3.4. Metabolic stability across multiple species

The in vitro metabolic stability of VBP6 and VBP15 was studied in different species of liver microsomes. Results show ⩾80% remaining at 1 h of VBP6 and VBP15 in human, dog, and monkey microsomes (Fig. 6). VBP6 had poor stability in mouse liver microsomes (34%) compared to VBP15 (88%). Both compounds showed poor stability in rat, presumably due to the high first pass clearance traditionally seen with glucocorticoids in rats.[19]

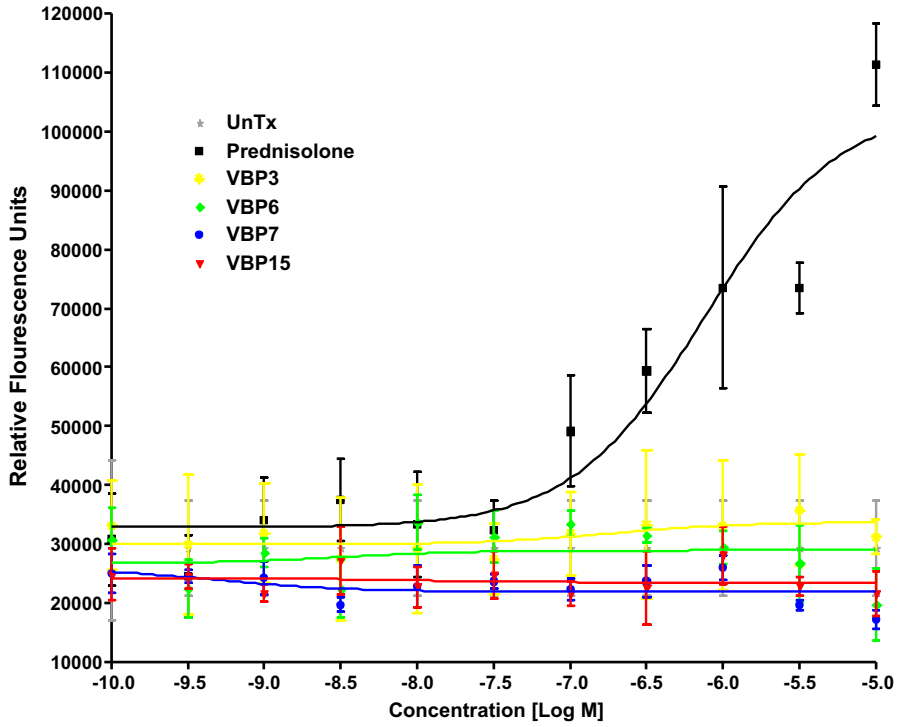


Figure 4. VBP compounds do not induce GRE-dependent gene transcription. A reporter construct with luciferase driven by glucocorticoid response elements (GREs) was studied, as previously described.[18] Prednisone induced luciferase expression, while none of the VBP series did. Standard deviations are shown.

Table 2
PK analysis of VBP6 and VBP15 following IV and PO administration in CD1 mice

Drug	Route	Dose (mg/kg)	Formulation	Cl_{obs} (mL/min/kg)	$T_{1/2}$ (h)	T_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h ng/mL)	AUC_{Inf} (h ng/mL)	MRT (h)	AUC/D (h ng/mL)	Vss_{obs} (L/kg)	*F* (%)
VBP6	IV	10	10% EtOH + 40% PEG400	48.2	0.667	0.0833	8.637	3.447	3.457	0.289	345	0.874	NA
	PO	50	30% Labrafil	NA	0.659	1.00	10.203	22.023	22.031	NA	440	NA	128
VBP15	IV	10	10% EtOH 10% DMSO 40% PEG400	18.8	0.354	0.0833	11.167	8.838	8.842	0.667	884	0.757	NA
	PO	50	30% Labrafil	NA	0.678	2.00	6.787	32.912	32.932	NA	658	NA	74.5

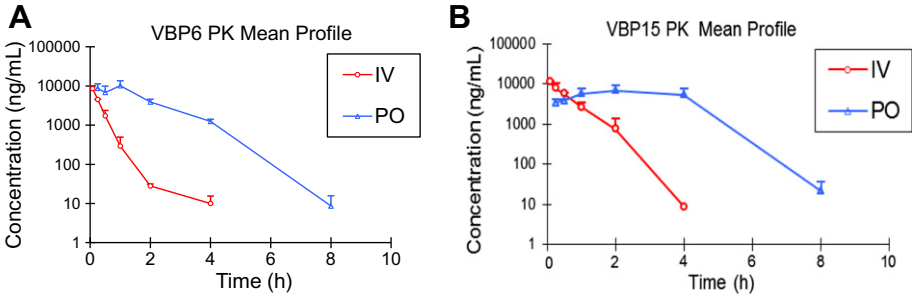


Figure 5. PK mean profiles for (A) VBP6 and (B) VBP15 in mice. Error bars indicate standard deviation.

3.5. Lead compound selection

Potential lead compounds were narrowed to VBP6 and VBP15 based on superior activities (high NF-κB inhibition, low cytotoxicity, high GR binding and GR translocation). PK data showed that VBP15 had a longer T_{max} and lower C_{max} across species, leading to a greater and preferred AUC compared to VBP6. Thus, VBP15 was selected as the lead compound for all further studies.

3.6. Absorption, distribution, metabolism and excretion (ADME) and pharmacokinetics of VBP15

We performed a series of ADME and pharmacokinetic studies to characterize our lead compound. Table 3 summarizes the results of these ADME experiments. PK results for VBP15 in rats indicate that it had a clearance of 20.2 mL/min/kg. The terminal half-life was 0.58 h. Volume of distribution (V_{ss}) was 0.77 L/kg, indicating that it did not

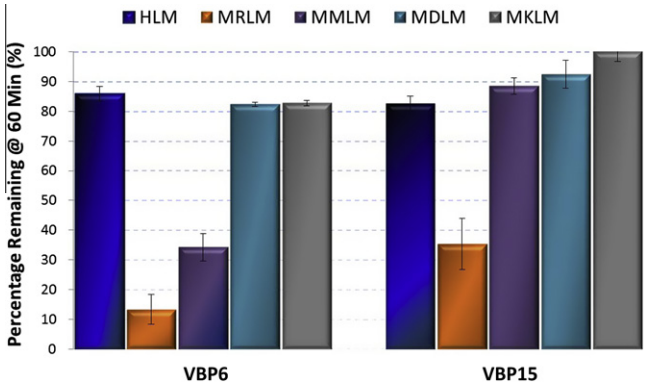


Figure 6. Metabolic stability of VBP6 and VBP15 in liver microsomes across multiple species. VBP15 shows better stability in rat and mouse microsomes compared to VBP6. HLM = human liver microsomes, MRLM = male rat liver microsomes, MMLM = male mouse liver microsomes, MDLM = male dog liver microsomes, MKLM = male monkey liver microsomes.

have high tissue accumulation. In the PO arm, C_{max} = 2543 ng/mL and percentage bioavailability (*F*%) was 47.8% (Table 4).

VBP15 in beagle dogs had medium-high clearance of 24.7 mL/min/kg. The terminal half-life was 5.42 h. Volume of distribution was 1.93 L/kg indicating that it had certain tissue distribution. In the PO arm, C_{max} = 814 ng/mL, and bioavailability (*F*%) was 53.2% (Table 4).

3.7. Metabolite identification

Human, monkey, dog, rat and mouse hepatocytes were incubated at a final concentration of 10 μM VBP15 for 2 and 4 h. Four metabolites were identified in the human and monkey hepatocytes, 3 were identified in the dog (peak 4 is the major metabolite), 5 metabolites were identified in rat (peak 3 is the major metabolite) and 2 in the mouse (Table 5).

3.8. CYP induction

There was no induction of CYP1A2 by VBP15 seen across the three donors therefore VBP15 is not considered an inducer of CYP1A2. VBP15 moderately induced CYP3A4 across the three donors (24–42%). This indicates that VBP15 is a potential inducer of CYP3A4, similar to other steroidal compounds.

4. Discussion

We have recently shown that an existing Δ9,11 steroidal compound (anecortave acetate) was able to bind and translocate the glucocorticoid receptor (GR) and inhibit anti-inflammatory pathways (NF-κB).[17] Based on these data we synthesized 20 new compounds not previously known to the literature, with the goal of carrying out a lead optimization program using the Δ9,11 backbone, in vitro screening assays included potent inhibition of NF-κB, potent translocation of the GR to the nucleus, loss of GRE-mediated transcriptional activity, and low cytotoxicity. We found that a C16-methyl group was important for NF-κB inhibitory activity, while larger alkyl groups (ethyl and butyl) either decreased activity or enhanced cytotoxicity of myogenic cells. Other optimizations of chemistry included a Δ1,4 in the A ring (improved metabolic stability), a Δ9,11 in the C ring, a methyl at C16, and either hydroxyl or hydrogen at C17. VBP15 was selected as the lead compound based on these findings. VBP15 also showed desirable ADME and PK properties. Like many steroids, VBP15 shows oral absorption in a target range acceptable for oral delivery. The drug can be formulated for PK and other studies in vehicles that are typical for related steroids. PK and metabolite work suggest that the primate and mouse will probably be the preferred species for PK.

Synthetic glucocorticoids are among the most commonly prescribed drugs due to their potent anti-inflammatory properties, and remain standard of care in many conditions such as arthritis, dermatitis, asthma, muscular dystrophy, and auto-immune disorders.[20–22] However, glucocorticoids have many off-target effects that together contribute to a relatively broad side effect profile. These include but are not limited to disruption of glucose metabolism, immune suppression, adrenal suppression, thymocyte apoptosis, osteoporosis, erythroblast proliferation, elevation of intraocular pressure, cataract, and mood changes. Many of these side effect profiles are associated with GRE-mediated transcriptional activities of steroids (classic glucocorticoid activity). We have shown that VBP15 lacks GRE-mediated transcriptional

Table 3
ADME properties of VBP15

In vitro properties	Units	Value & class	Target range
Solubility (pH, media)	(μM)	187.18 μM	>60
Stability—microsomes	$t_{1/2}$ (min)	Human = 82% Rat = 35.4% Mouse = 88.8% Dog = 92.5% Monkey = 100%	>30
Stability—plasma (species)	% Remaining at 1 h	88.06%	>80%
CYP450 Inhibition (2C9,2D6,3A4)	% Inhibition at 10 mM IC_{50} (mM)	1.9%, 7.9%, 15.3% >50 μM for all	<40% >10
Permeability—Caco-2	P_{app} (a–b, 10^{-6} cm/s) Efflux ratio	11.2 0.61	>10 <2
hERG— (method)	IC_{50} (mM)	20 μM	>10
Free C_{max}—plasma	Total C_{max} (mM) $^{*}F_{u,\ plasma}$	0.12	
Ames test	Positive/negative	Negative	Negative
Micronucleus test	Positive/negative	Negative	Negative
Blood Brain Barrier (rats)[a]	Ratio (brain/plasma) (h)	0.25 = 0.648 0.5 = 0.620 1.0 = 0.614 2.0 = 0.593 4.0 = 0.583 6.0 = 0.581 8.0 = 0.433	

[a] The brain concentration was not corrected for vascular content.

Table 4
PK properties of VBP15 across species

PK properties	Units	Mouse 10 mpk IV 50 mpk PO	Rat 10 mpk IV 50 mpk PO	Dog 10 mpk IV 30 mpk PO	Target range
$t_{1/2}$	h	0.354 (IV) 0.678 (PO)	0.58 (IV) 2.29 (PO)	5.42 (IV) 2.25 (PO)	>3
$AUC_{0-\infty,\ total,\ unbound}$	h ng/mL	8842 (IV) 32932 (PO)	8339 (IV) 19937 (PO)	6791 (IV) 10844 (PO)	>500 (PO)
CL	mL/min/kg	18.8 (IV)	20.2 (IV)	24.7 (IV)	<25% HBF
$C_{max,\ total,\ unbound}$	ng/mL (nM)	11167 (IV) 6787 (PO)	2543 (PO)	814 (PO)	
T_{max}	h	0.083 (IV) 2.0 (PO)	4.00 (PO)	6.00 (PO)	
V_{ss}	L/kg	0.757 (IV)	0.770 (IV)	1.93 (IV)	
F	%	74.5	47.8	53.2	>20%

Table 5
VBP15 metabolites identified across species

Peak	Rt	Expected	Mass	Biotransformation	Metabolites in hepatocytes				
no.	(min)	m/z	shift		Human	Monkey	Dog	Rat	Mouse
1	5.52	371.2	16	Oxidation	+	+	+	+	+
2	6.37	373.2	18	Oxidation + hydrogenation	+	+	–	+	–
3	7.78	371.2	16	Oxidation	–	–	–	+++	–
4	9.69	369.2	14	Methylation	+	+	+++	+	+
5	10.42	355.2	0	Parent drug	+++	+++	+	+	+++
6	12.86	357.2	2	Hydrogenation	+	+	+	+	–

activity, although it inhibits NF-κB and causes translocation of the GR to the nucleus. We should note that classic glucocorticoids result in nuclear translocation as ligand/GR dimmers able to bind GRE elements (transactivation properties), as well as ligand/GR complexes with NF-κB transcriptional complexes that target NF-κB promoter elements (transrepression properties). VBP15 appears to disassociate these two subactivities, with GR translocation likely due to NF-κB transcriptional complexes. Thus, we expect that preclinical studies will show superior side effect profiles of VBP15 compared to other steroid-based anti-inflammatories, as we have previously shown for anecortave acetate.[17]

The extensive effect profiles of pharmacological glucocorticoids often limit prescription despite proven efficacy, particularly in children. For example, in Duchenne muscular dystrophy, daily prednisone is considered standard of care in some countries, but the efficacy if offset by increased bone fragility, mood changes, weight gain, and muscle catabolic pathways.[23–26] Thus, there is non-adoption of this standard in some countries, and relatively poor adherence even in those countries adopting daily glucocorticoids.[27] The VBP15 described here is being developed for Duchenne muscular dystrophy as the initial indication, in collaboration with the Muscular Dystrophy Association Venture Philanthropy, and National Institutes of Health Therapeutics for Rare and Neglected Disease program. It is our hope that VBP15 not only provides a superior side effect profile relative to prednisone, but will also improve efficacy by opening the therapeutic window (allowing increased dosing relative to prednisone).

Acknowledgments

Supported in part by Grants from the Congressionally Directed Medical Research Program of the US Department of Defense (W81XWH-09-1-0218; W81XWH-11-1-0754), Muscular Dystrophy Association Venture Philanthropy (Phase 1 and Phase 2), Foundation to Eradicate Duchenne, NIH Wellstone Muscular Dystrophy Research Center (1U54HD053177-01A1), and NIH Therapeutics for Rare and Neglected Disease (TRND) program.

References and notes

1. Hall, E. D.; Braughler, J. M.; McCall, J. M. *Prog. Clin. Biol. Res.* **1990**, *361*, 351–362.
2. Bracken, M. B.; Shepard, M. J.; Holford, T. R.; Leo-Summers, L.; Aldrich, E. F.; Fazl, M.; Fehlings, M.; Herr, D. L.; Hitchon, P. W.; Marshall, L. F.; Nockels, R. P.; Pascale, V.; Perot, P. L., Jr.; Piepmeier, J.; Sonntag, V. K.; Wagner, F.; Wilberger, J. E.; Winn, H. R.; Young, W. *JAMA* **1997**, *277*, 1597–1604.
3. Bracken, M. B.; Shepard, M. J.; Holford, T. R.; Leo-Summers, L.; Aldrich, E. F.; Fazl, M.; Fehlings, M. G.; Herr, D. L.; Hitchon, P. W.; Marshall, L. F.; Nockels, R. P.; Pascale, V.; Perot, P. L., Jr.; Piepmeier, J.; Sonntag, V. K.; Wagner, F.; Wilberger, J. E.; Winn, H. R.; Young, W. *J. Neurosurg.* **1998**, *89*, 699–706.
4. Lanzino, G.; Kassell, N. F.; Dorsch, N. W.; Pasqualin, A.; Brandt, L.; Schmiedek, P.; Truskowski, L. L.; Alves, W. M. *J. Neurosurg.* **1999**, *90*, 1011–1017.
5. Lanzino, G.; Kassell, N. F. *J. Neurosurg.* **1999**, *90*, 1018–1024.
6. van der Worp, H. B.; Kappelle, L. J.; Algra, A.; Bär, P. R.; Orgogozo, J. M.; Ringelstein, E. B.; Bath, P. M.; van Gijn, J. *Neurology* **2002**, *58*, 133.
7. Kavanagh, R. J.; Kam, P. C. *Br. J. Anaesth.* **2001**, *86*, 110–119.
8. Wendler, A.; Wehling, M. *Steroids* **2010**, *75*, 619–623.
9. Flessas, I. I.; Papalois, A. E.; Toutouzas, K.; Zagouri, F.; Zografos, G. C. *J. Surg. Res.* **2011**, *166*, 265–274.
10. McNatt, L. G.; Weimer, L.; Yanni, J., et al *J. Ocul. Pharmacol. Ther.* **1999**, *15*, 413–423.
11. Penn, J. S.; Rajaratnam, V.; Collier, R. J.; Clark, A. F. *Invest. Ophthalmol. Vis. Sci.* **2001**, *42*, 283.
12. D'Amico, D. J.; Goldberg, M. F.; Hudson, H.; Jerdan, J. A.; Krueger, D. S.; Luna, S. P.; Robertson, S. M.; Russell, S.; Singerman, L.; Slakter, J. S.; Yannuzzi, L.; Zilliox, P. *Ophthalmology* **2003**, *110*, 2372. discussion 2384–5.
13. Slakter, J. S.; Bochow, T. W.; D'Amico, D. J.; Marks, B.; Jerdan, J.; Sullivan, E. K.; Robertson, S. M.; Slakter, J. S.; Sullins, G.; Zilliox, P. *Ophthalmology* **2006**, *113*, 3–13.
14. Stalmans, I.; Callanan, D. G.; Dirks, M. S.; Moster, M. R.; Robin, A. L.; Van Calster, J.; Scheib, S. A.; Dickerson, J. E., Jr.; Landry, T. A.; Bergamini, M. V. *J. Ocul. Pharmacol. Ther.* **2012**, *28*, 559–565.
15. Robin, A. L.; Suan, E. P.; Sjaarda, R. N.; Callanan, D. G.; Defaller, J. *Arch. Ophthalmol.* **2009**, *127*, 173–178.
16. Robin, A. L.; Clark, A. F.; Covert, D. W.; Krueger, S.; Bergamini, M. V.; Landry, T. A., ; Dickerson, J. E., Jr.; Scheib, S. A.; Realini, T.; Defaller, J. M.; Cagle, G. D. *Am. J. Ophthalmol.* **2009**, *147*, 45.
17. Baudy, A. R.; Reeves, E. K.; Damsker, J. M.; Heier, C.; Garvin, L. M.; Dillingham, B. C.; McCall, J.; Rayavarapu, S.; Wang, Z.; Vandermeulen, J. H.; Sali, A.; Jahnke, V.; Duguez, S.; DuBois, D.; Rose, M. C.; Nagaraju, K.; Hoffman, E. P. *J. Pharmacol. Exp. Ther.* **2012**, *343*, 225–232.
18. Baudy, A. R.; Saxena, N.; Gordish, H.; Hoffman, E. P.; Nagaraju, K. *Int. Immunopharmacol.* **2009**, *9*, 1209–1214.
19. Haughey, D. B.; Jusko, W. J. *J. Pharm. Sci.* **1992**, *81*, 117–121.
20. Bijlsma, J. W. *Rheumatology (Oxford)* **2012**, *51*, iv9.
21. Butterfield, J. H.; Weiler, C. R. *Semin. Hematol.* **2012**, *49*, 182–191.
22. LeHoang, P. *Dev. Ophthalmol.* **2012**, *51*, 7–28.
23. Sandri, M.; Sandri, C.; Gilbert, A.; Skurk, C.; Calabria, E.; Picard, A.; Walsh, K.; Schiaffino, S.; Lecker, S. H.; Goldberg, A. L. *Cell* **2004**, *117*, 399–412.

24. Zheng, B.; Ohkawa, S.; Li, H.; Roberts-Wilson, T. K.; Price, S. R. *FASEB J.* **2010**, *24*, 2660–2669.
25. Gokulakrishnan, G.; Estrada, I. J.; Sosa, H. A.; Fiorotto, M. L. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **2012**, *302*, R1143–R1152.
26. Reeves, E. K.; Rayavarapu, S.; Damsker, J. M.; Nagaraju, K. *Endocr. Metab. Immune Disord. Drug Targets* **2012**, *12*, 95–103.
27. Hoffman, E. P.; Reeves, E.; Damsker, J.; Nagaraju, K.; McCall, J. M.; Connor, E. M.; Bushby, K. *Phys Med Rehabil Clin N Am.* **2012**, *23*, 821–828.

Glucocorticoid Analogues: Potential Therapeutic Alternatives for Treating Inflammatory Muscle Diseases

Erica K.M. Reeves[3,#], Sree Rayavarapu[1,2,#], Jesse M. Damsker[3] and Kanneboyina Nagaraju[1,2,3,*]

[1]*Center for Genetic Medicine Research, Children's National Medical Center, Washington DC;* [2]*The George Washington University, Institute of Biomedical Sciences, Washington DC;* [3]*ReveraGen BioPharma, Inc. Rockville, MD, USA*

Abstract: Glucocorticoids (GCs) have been prescribed to treat a variety of diseases, including inflammatory myopathies and Duchenne muscular dystrophy for over 50 years. However, their prescription remains controversial due to the significant side effects associated with the chronic treatment. It is a common belief that the clinical efficacy of GCs is due to their transrepression of pro-inflammatory genes through inhibition of inflammatory transcription factors (*i.e.* NF-κB, AP-1) whereas the adverse side effects are attributed to the glucocorticoid receptor (GR) -mediated transcription of target genes (transactivation). The past decade has seen an increased interest in the development of GR modulators that maintain the effective anti-inflammatory properties but lack the GR-dependent transcriptional response as a safe alternative to traditional GCs. Many of these analogues or "dissociative" compounds show potential promise in *in vitro* studies but fail to reach human clinical trials. In this review, we discuss molecular effects of currently prescribed GCs on skeletal muscle and also discuss the current state of development of GC analogues as alternative therapeutics for inflammatory muscle diseases.

Keywords: Glucocorticoids, inflammatory muscle diseases, muscular dystrophy, myositis.

INTRODUCTION

Glucocorticoids (GCs) are the first line of drugs in the clinical management of idiopathic inflammatory muscle diseases [Polymyositis (PM) and Dermatomyositis (DM)] and genetic muscle diseases such as Duchenne Muscular Dystrophy (DMD). Generally, GCs are believed to be effective in these diseases because of their anti-inflammatory and immunosuppressive properties. Even though the inflammatory component is common, these idiopathic inflammatory and DMD muscle diseases differ significantly in their histological and clinical features which are summarized below. In addition, features of some major muscle diseases with inflammatory component are described in Table **1**.

Duchenne Muscular Dystrophy

DMD is an X-linked myopathy caused by a mutation in the dystrophin gene and is characterized by general weakness and muscle wasting. Initial onset of disease is in pelvic girdle and further progresses towards the distal and respiratory muscles. The lack of dystrophin in the sarcolemma leads to skeletal muscle fiber necrosis and initiates the regeneration, degeneration phases in the affected muscle. However, the progression of the disease makes the normal regenerative capacity of muscle insufficient to replace necrotic muscle fibers leading to subsequent fibrosis and severely impaired muscle function [1]. Currently, the underlying pathogenic mechanisms of severe muscle damage in dystrophinopathies are not completely understood. However, it is generally thought that membrane defects due to lack of dystrophin and further mechanical injury due to general activity initiate chronic inflammatory pathways and promote dystrophic pathology [2, 3]. Inflammation in dystrophic muscle is characterized by the activated immune cell infiltrates and up-regulated inflammatory mediators (*e.g.*, cytokines and chemokines) [4, 5]. Immune cell infiltration is throughout the perivascular, perimysial, and endomysial regions of dystrophic skeletal muscle with the infiltrate predominantly containing macrophages, and $CD4^+$ T-cells, with minimal $CD8^+$ T-cells and B-cells [6, 7]. In addition, mast cells, eosinophils, and neutrophils also contribute to muscle fiber wasting in dystrophic pathology [8, 9]. The enhanced expression of MHC class I molecules, on muscle fibers invaded by inflammatory cells, and on necrotic and regenerating fibers in dystrophic muscle indicate that immune effector mechanisms also contribute to the disease pathology in DMD [10, 11]. Most of these immune mechanisms are proposed to act *via* the activation of pro-inflammatory NF-κB pathway in dystrophic muscle [3, 7]. Taken together, these findings indicate the substantial role of inflammation and its mediators in dystrophic pathology and hence support the use of GCs as potential therapeutic agents.

Idiopathic Inflammatory Muscle Diseases (IIMs)

Idiopathic inflammatory muscle diseases are characterized clinically by symmetric proximal muscle weakness and histologically by mononuclear cell infiltration, fibrosis, and fat deposition in muscle tissue. In general, muscle weakness in IIMs is thought to be the result of tissue damage caused by an autoimmune response. Both humoral (auto-antibodies) and cell mediated (cytotoxic T lymphocytes)

*Address correspondence to this author at the Director; Murine Drug Testing Facility, Associate Director; Research Center for Genetic Medicine, Associate Professor; Institute of Biomedical sciences, The George Washington University, Children's National Medical Center, 111 Michigan Ave. N.W. Washington DC 20010, USA; Tel: 202-476-6220; Fax: 202-476-6014; E-mail: knagaraju@cnmcresearch.org

[#]First co-author

2212-3873/12 $58.00+.00

Table 1. Characteristics of some major muscle diseases with inflammatory component.

Disease	Features
Duchenne muscular dystrophy[1]	Mutation in dystrophin gene leads to elevated levels of serum creatine kinase (CK), muscle weakness, respiratory insufficiency and cardiomyopathy. Dystrophic muscles show infiltration of B cells, T cells and macrophages.
Dysferlinopathies (LGMD2B and Miyoshi myopathy)	Mutation in dysferlin gene leads to progressive proximal (LGMD2B) or distal (Miyoshi) muscle weakness with elevated serum CK levels. Histologically muscles show infiltration of $CD8^+$, $CD4^+$ T cells and macrophages.
Fascioscapulohumeral muscular dystrophy	Deletion in D4Z4 repeats leads to mild or moderate levels of serum CK levels, initial weakness in proximal muscles which progresses to distal muscles in later stages. Affected muscles show perivascular mononuclear cell infiltration.
Polymyositis[1]	Symmetric proximal muscle weakness with elevated levels of serum muscle enzymes and autoantibodies (anti-Jo1). Myopathic muscle shows endomysial infiltration of mononuclear cells ($CD8^+$ T cells, macrophages and B cells) and expression of MHC class-I molecules.
Dermatomyositis[1]	Symmetric proximal muscle weakness with skin involvement and elevated levels of serum muscle enzymes and autoantibodies (anti-Mi2). Muscle biopsies show perivascular and epimysial infiltration of mononuclear cells ($CD4^+$ T cells, B-cells and macrophages).
Inclusion body myositis	Predominantly affects males over 50 years of age; muscle weakness with elevated serum muscle enzymes. Affected muscle tissue shows MHC class-I expression with mononuclear cell infiltration.

[1]Glucocorticoids are shown to be effective in these diseases.

immune responses are reported to be involved in the pathogenesis and are well characterized. Myositis specific auto-antibodies are found in more than 50% of myositis patients [12]. For example, anti-synthetase autoantibodies [anti-histidyl tRNA synthetase (Jo-1)] are common in PM patients and anti-helicase antibodies (Mi-2α & β) are strongly associated with DM patients. However, the precise role of these auto antibodies in the pathogenesis of myositis is not well known. At the cellular level, PM patients show endomysial distribution of inflammatory cell infiltrates with predominantly $CD8^+$ T cells and macrophages. In contrast, DM patients predominantly show perivascular distribution of inflammatory cells which largely include $CD4^+$ T cells, macrophages, and dendritic cells. Occasionally, DM patients also display involvement of skin and vasculature where B cells and complement cascade activation are thought to be responsible for the pathology [12]. Another striking feature of IIM is the early widespread appearance of MHC class-I molecules in the myopathic muscle whereas normal muscle does not constitutively express them. Evidence suggests that MHC class-I molecules may mediate muscle fiber damage and dysfunction in myositis [13]. Over expression of MHC class-I molecules might cause their retention in the endoplasmic reticulum (ER) of skeletal muscle fibers and induce NF-κB mediated ER stress pathways, possibly contributing to muscle fiber damage in IIMs [14, 15]. Thus, immune mechanisms *via* pro-inflammatory NF-κB pathway are thought to play a potential role in the underlying myopathic pathology.

GLUCOCORTICOIDS IN INFLAMMATORY MUSCLE DISEASES

Even though it is not fully understood how GCs exert beneficial effects on the diseased muscle, they are being used to treat inflammatory muscle diseases because of their strong anti-inflammatory and immunosuppressive properties. Synthetic GCs (*i.e.* prednisone) are the standard-of-care for DMD as it has been shown to improve muscle strength and delay the disease progression of disease in boys [16]. Myositis patients are also often treated with high-dose synthetic GCs for 4 to 12 weeks and sometimes in combination with other cytotoxic drugs (methotrexate) [17].

The most well-characterized mechanism of action for GCs is the genomic response whereby GCs bind to the soluble glucocorticoid receptor (GR) with subsequent translocation of the ligand/receptor complex into the nucleus, followed by binding to glucocorticoid response elements (GREs) in the promoter regions of target genes (*i.e.* genes involved in gluconeogenesis and inflammation), altering transcription (transactivation). In addition, the ligand/ receptor complex can also bind to transcription factors and either compete with or prevent their association with DNA thereby inhibiting the transcription of their target genes (*e.g.*, activator protein 1 (AP-1) and NF-κB) (transrepression). It is widely believed that many of the desirable effects of GCs on skeletal muscle are due to transrepression (*i.e.* suppression of pro-inflammatory mediators) whereas the adverse effects (*i.e.* hyperglycemia, weight gain) are mediated by transactivation [18].

Glucocorticoids also exhibit non-genomic actions that occur too rapidly to be attributed to a genomic response. These actions include intercalation into the cellular membranes, modulation of the function of membrane associated proteins, alteration of the membrane permeability, and calcium signaling [19]. Glucocorticoids also affect oxidative phosphorylation, ATP production and protein-protein signaling processes (*i.e.* MAPK pathway) [20, 21]. Despite the beneficial effects, GCs exhibit significant adverse side effects upon long-term use. In this review, we summarize the current knowledge of the most important GC-mediated beneficial and adverse effects on diseased skeletal

muscle from a molecular perspective and discuss the development of alternative therapeutics for the treatment of inflammatory muscle diseases.

Beneficial Effects of Glucocorticoids in DMD and IIMs

The beneficial effects of GCs in patients with DMD have been known for over 20 years [22]. Despite the fact that GCs have been shown to be catabolic in normal muscle, DMD patients show improved muscle strength and delayed progression of disease upon GC treatment [23]. To better understand the molecular effects of GCs on dystrophic muscle, studies often utilize the dystrophin deficient mouse model (*mdx*). In this animal model, it was shown that 8 weeks of prednisolone treatment increased specific force of the EDL muscle and that this increase in force is attributed to the decrease in muscle mass and muscle fiber cross sectional area. Prednisolone treatment decreased the centrally nucleated fibers in the dystrophic muscle and suggested this feature as an improvement in the histological phenotype [24]. In contrast to its effect on dystrophic muscle, prednisolone treatment did not increase force or reduce the force deficit in acutely injured normal muscle, indicating that the action of prednisolone on dystrophic muscle is different in comparison to acutely injured normal muscle [24]. One explanation for this conundrum might be that an acute injury and the subsequent recovery processes of muscle are systematically regulated events whereas the dystrophic muscle is undergoing cycles of necrosis and degeneration due to the continuous mechanical damage of the susceptible (dystrophin deficient) muscle membrane. Similar to DMD patients, the treatment of PM and DM patients with GCs also resulted in the amelioration of muscle disease; however, complete recovery is rarely seen. Existing literature links the beneficial effects of GCs to their immunosuppressive and potent NF-κB inhibitory actions [15]. However, more targeted mechanistic studies explaining the molecular effect of GCs on myopathic muscle are needed.

Adverse Effects of Glucocorticoids

Chronic administration of GCs result in adverse effects whose underlying molecular mechanisms are complex and poorly understood. Glucocorticoids induce skeletal muscle atrophy by reducing protein synthesis and by enhancing protein degradation. Glucocorticoids inhibit p70 ribosomal S6 protein kinase and in turn reduce protein synthesis. Furthermore, GCs induce muscle proteolysis *via* the activation of two major cellular proteolytic systems: ubiquitin proteasome and lysosomal systems [25]. Administration of GCs reduces the production of insulin-like growth factor I (IGF-I), a muscle anabolic growth factor, by the skeletal muscle which might in turn contribute to GC-induced muscle atrophy. Evidence indicates that GCs enhance the production of myostatin, a catabolic growth factor, which may also play a critical role in GC-induced muscle atrophy [26]. REDD1 and KLF15 were reported as direct target genes of the GR in skeletal muscle and have been reported that a mutually exclusive crosstalk between GR and mTOR, might be responsible for fine-tuning of muscle volume and further muscle wasting [27]. Recently, our group also showed that chronic administration of GCs activate fibrotic genes (*i.e.* genes interacting with collagen deposition, abnormal inflammatory response) in the dystrophic skeletal muscle [28]. Glucocorticoids are also known to induce other potential side effects in essential organ systems such as the cardiovascular and central nervous systems upon chronic administration. These adverse effects include osteoporosis, glucocorticoid induced diabetes, glaucoma etc and are thoroughly reviewed elsewhere [29]. Further, the gene expression profiles of prednisolone treated *mdx* mice muscle showed changes in energy metabolism and proteolysis, in addition to changes in MAPK and VEGF signaling, suggesting that prednisolone has multiple effects on several pathways. Therefore, it remains to be seen whether these pathways contribute to the beneficial or adverse effects in dystrophin deficient skeletal muscle.

THE USE OF MORE TARGETED ANTI-INFLAMMATORIES IN INFLAMMATORY MUSCLE DISORDERS

Despite the effectiveness, chronic administration of GCs in inflammatory muscle diseases is restricted due to adverse side effects. Therefore, several other alternative immuno-suppressive agents have been tried in these diseases to ameliorate chronic inflammation and to facilitate GC tapering, but there is no agreement as to the best regimen of immunosuppressive drugs to use in myopathic patients. The choice of agent typically depends on factors such as disease severity and the relative efficacy/safety profile of the drug. Some of the anti-inflammatory drugs tested for efficacy in IIMs and DMD are listed in Table **2**. Mixed results were reported regarding the usefulness of these agents as potential treatment regimens for inflammatory muscle diseases. Nevertheless, these drugs were also reported to have significant side effects such as bone marrow suppression and hepatic toxicity [30].

DISSOCIATIVE NONSTEROIDAL COMPOUNDS

As previously mentioned, it is widely accepted that the positive anti-inflammatory effects of GCs are mediated by their transrepression properties whereas the adverse side effects are mediated *via* transactivation. Significant side effect profile of these compounds lead to the development of several new compounds that have dissociated transrepressive properties of traditional steroids from the transactivational properties. The majority of the research into 'dissociative' compounds has been performed in models for indications other than muscle diseases but still provide valuable insight into how well these compounds are at reducing or eliminating systemic side effects.

AL-438

In 2003, collaboration between Abbott Laboratories and Ligand Pharmaceuticals resulted in the development of a series of novel quinolone-based nonsteroidal GC agonists [31, 32]. AL-438 was created by modifying the progestin scaffold, and was found to bind selectively to the GR with high affinity compared to other nuclear receptors [31]. *In vitro* experiments showed AL-438 effectively transrepressed the production of TNF-α and IL-1β- induced IL-6 and E-

Table 2. Targeted anti-inflammatory drugs used in genetic and autoimmune muscle diseases.

Drug	Mechanism of Action	Therapeutic Efficacy[1]	References
Methotrexate	Prevent chemotaxis and the release of pro-inflammatory mediators	DM & Adult Inflammatory myositis	[68, 69]
Azathioprine	Immunosuppression & effect on neuromuscular transmission	DM & PM Not effective in DMD	[70, 71]
Cyclosporine	Inhibits T cell activation and subsequent cytokine production	PM & Juvenile DM Not effective in DMD	[70, 72]
Intravenous immunoglobulin	Immunomodulatory effects, reduction in cytokines, MHC class I and ICAM-1 expression	DM	[30, 73]
Infliximab	TNF- α inhibition	DM & PM	[74]
Rituximab	Inhibits B-cells	Refractory DM & PM	[75]

[1]Therapeutic efficacy was tested and reported to be efficacious in the disease mentioned
DM: Dermatomyositis
PM: Polymyostis
DMD: Duchenne muscular dystrophy.

selectin levels. Its transactivational properties where shown to be approximately 30% less potent in activation of GRE-mediated transcription of aromatase but just as potent in activation of tyrosine aminotransferase transcription (TAT) when benchmarked against prednisolone. In order to assess the effect of AL-438 on bone reabsorption, osteocalcin inhibition (a marker for GC-mediated osteoporosis) was measured. While prednisone significantly decreased osteocalcin mRNA levels in the osteoblast (MG63) cell line, AL-438 showed no change suggesting less effect on bone reabsorption.

To determine the effect of AL-438's dissociative properties *in vivo*, the carrageenan-induced paw edema assay (CPE) rat model of acute inflammation, and the adjuvant-induced arthritis (AIA) rat model of chronic inflammation were used [31]. In the CPE model, prednisolone and AL-438 were similarly effective in inhibiting paw edema. In the AIA rat model, both compounds were equally effective in inhibiting paw edema though AL-438 had a lower potency compared to prednisolone (ED50s= 9 mg/kg and 1 mg/kg respectively). Further, there was a difference in the grooming behavior, activity and overall health of AL-438 versus prednisolone treated rats. AL-438-treated rats maintained similar levels of behavior and health as normal non-adjuvant injected rats whereas prednisolone-treated rats showed signs of stress and disease suggesting AL-438's superiority over prednisolone.

Coghlan *et al.* (2003) investigated the possible side effects of AL-438 on glucose metabolism and osteoporosis associated with GCs. They found that AL-438 did not induce GC-mediated increase in blood glucose suggesting that it does not cause hyperglycemia. They also found that AL-438 reduced the impact on bone as determined by cancellous mineral apposition rate and periosteal bone formation rate supporting their *in vitro* data. Other studies have further confirmed the reduced effect on bone where AL-438 did not share the dexamethasone-induced reduction of chondrocyte cell proliferation and proteoglycan synthesis [31]. These findings suggest that the dissociation of AL-438's anti-inflammatory effects from the bone growth side effects result in a compound with a more desirable therapeutic profile than that of traditional GCs [33].

ZK Compounds

ZK 216348, a nonsteroidal GR ligand created by Bayer Schering Pharma, is a pentanoic acid 4-methyl-1-oxo-1H-2,3-benzoxazinamide [34]. This compound has been shown to successfully dissociate transrepression from trans-activation *in vitro*. ZK 216348 inhibited IL-8 secretion in human acute monocytic leukemia cells with a two-fold less potency than prednisolone whereas it was 60-fold less potent in the induction of TAT in liver hepatoma cells but was as effective, albeit less potent, in TNF-α and IL-12 production in human peripheral blood mononuclear cells suggesting the maintenance of anti-inflammatory properties. Additionally it was shown that, similar to AL-438, ZK 216348 also did not affect osteoblasts in *in vitro* experiments suggesting a reduced effect on bone [35]. This dissociation profile of ZK216348 was carried over to *in vivo* experiments and was found to be effective in reducing ear inflammation in the croton oil-induced ear inflammation model of both rats and mice. The effect was comparable to prednisone but with a significantly reduced efficacy in rats *via* topical administration [34]. ZK 216348 has a superior side effect profile that lacked hyperglycemia, spleen involution, and skin atrophy (though to a lesser extent); however, ACTH suppression was similar for both ZK 216348 and prednisolone.

Another ZK compound in this series, ZK 245186 maintained a similar *in vitro* and *in vivo* dissociative profile as ZK 216348 regarding; GR binding specificity, TAT induction, blood glucose level, and spleen involution [36]. Further *in vitro* characterization demonstrated that ZK 245186 inhibits LPS-induced secretion of IL-12p40 and phytohemagglutinin stimulated INF-γ in human peripheral blood mononuclear cells. *In vivo* testing using the mouse and

rat models of irritant contact dermatitis and allergic contact dermatitis showed that this compound exerted potent anti-inflammatory activity. Pharmacokinetic analysis showed ZK 245186 to be metabolically unstable across multiple species, high hepatic clearance, a short half-life after intra venous injection, and low bioavailability after topical administration. These findings suggest that ZK 245186 would be well suited for topical treatment with a therapeutic index similar to that of traditional steroids but with an improved safety profile. ZK 245186 is currently in Phase II clinical trials for Atopic Dermatitis.

LGD-5552

LGD-5552, a C5-benzylidene developed by Ligand Pharmaceuticals, shares a similar scaffold as AL-438 [37]. Competitive binding experiments showed that LGD-5552 has a high affinity for the GR with little to no cross-reactivity with androgen and progesterone receptors [37, 38]. However, it did have antagonist activity with the mineralocorticoid receptor as opposed to traditional GCs. These data suggest that LGD-5552 might have effects on urinary output, sodium and potassium levels, and blood pressure *in vivo*. *In vitro* experiments indicate that LGD-5552 retains the agonistic properties, in repressing IL-1β/TNF-α induced activation of E-selectin and IL-6 transcription similar to steroids [37, 38]. Both prednisolone and LGD5552 inhibited Cox2 and Apolipoprotein CIII mRNA levels. LGD-5552 was proven to be a potent *in vivo* anti-inflammatory in CIA and AIA animal models with significant decrease in paw volume and arthritis disease score [37, 38]. Furthermore, analysis of inflammation-related genes showed that LGD-5552 differentially regulated IL-10 and MCP-1 compared to prednisolone [38].

LGD-5552 improved side effect profiles upon administration when compared to prednisolone-treated animals. LGD-5552 did not increase percent body fat as seen with prednisolone [37, 38]. Bone formation was significantly decreased with prednisolone at all doses tested whereas there was no change in LGD-5552-treated animals except at the highest dose tested. In rats, LGD-5552 was less active at lower doses compared to prednisolone in increasing arterial blood pressure and less potent in reducing body weight, thymus weight and adrenal weight.

Compound A

Compound A (CpdA) is a stable analog of the hydroxy phenyl aziridine precursor isolated from the Namibian shrub *Salsola tuberculatiformis* Botschantzev and is the first dissociated compound described to derive from a natural source. It has a high affinity to the GR and upon binding, favors a monomeric GR conformation [39, 40]. CpdA-induced GR nuclear translocation but did not activate the GRE-dependent transcription of matrix metalloproteinase-1 (MMP-1), MMP-3, TAT, human placental alkaline phosphatase, procollagen C-endopeptidase enhancer 2 and glucocorticoid-induced leucine zipper (GILZ) [39-42, 44-47]. CpdA was able to effectively transrepress downstream TNF-α induced and NF-κB-dependent targets including IL-6, E-selectin, intracellular adhesion molecule, IL-8, MMP-13, glucocorticoid-binding globulin, pro-opiomelanocortin and gonadotropin-releasing hormone.

In vivo analysis of CpdA demonstrated clear anti-inflammatory properties in two inflammatory animal models (the zymosan-induced inflamed paw model and CIA) and two neuroinflammation models [Experimental Autoimmune Encephalomyelitis (EAE) and Experimental Autoimmune Neuritis (EAN)]. In addition to the potent anti-inflammatory properties of CpdA in the zymosan-induced inflamed paw and CIA models, there was an absence of hyperglycemia and hyperinsulinemia [39, 40]. In EAN, a model for human acute inflammatory demyelinating polyradiculoneuropathies, anti-inflammatory M2 macrophages, CD4+ T cells, and Th2 cytokines were increased whereas Th1 and Th17 cytokines were repressed in the lymph nodes. CpdA-treated animals also did not exhibit hyperglycemia [43]. In the EAE model for multiple sclerosis, CpdA ameliorated disease severity and clinical symptoms. Analysis of spinal cord tissue revealed reduced levels of inflammatory cells, cytokines and chemokines, as well as less NF-κB binding to the IL-6 promoter [41, 44]. Side effect profiles in these various models showed that CpdA do not cause hyperglycemia and liver toxicity; further, it has no effect on thymus weight [41, 44]. Interestingly, CpdA did not suppress the hypothalamic pituitary adrenal (HPA) axis thus providing another advantage over traditional GCs. However, in the EAE model, there is a narrow therapeutic window for CpdA due to its instability. High doses of CpdA led to the formation of toxic aziridine levels resulting in apoptosis and death [41]. This may likely have some adverse effects in human and would need to be resolved before clinical trials become an option.

DISSOCIATIVE STEROIDAL COMPOUNDS

RU Compounds

A series of RU-steroidal compounds were discovered by Roussel-Uclaf that retained transrepression of AP-1 and NF-κB but did not or only weakly possessed transactivation properties *in vitro* [45]. RU-compounds are shown to be weak stimulators of receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPN) in human osteoblastic cells and indicated that these compounds might cause less bone loss compared to traditional GCs. One of the RU-compounds (24858) was tested in an *in vivo* rat model of lung edema where it was found be just as potent as budesonide and prednisolone in terms of its anti-inflammatory properties. However, RU 24858 also induced side effects seen with traditional GCs, such as weight loss, osteopenia and thymus involution within seven days of administration [46]. The authors concluded that dissociation of transrepression and transactivation does not improve the *in vivo* therapeutic ratio of GCs and that *in vitro* dissociation does not necessarily eliminate the *in vivo* side effects. It has been suggested that the steroidal structure is metabolized *in vivo* in a manner that causes it to behave as a traditional GC [47, 48]. It has also been suggested that *in vitro* experiments looking at the GRE-transcriptional response use simple GRE luciferase reporting systems that do not accurately assess the response to complex GREs *in vivo* [49].

Table 3. Potential alternative therapeutic dissociative compounds for treating genetic and autoimmune inflammatory muscle diseases.

Drug	*In Vitro*[1]	*In Vivo*[2]	Human[3]	References
Dissociative Non-steroidal Compounds				
AL-438	+	+	NT	[31-33]
ZK compounds	+	+	Phase II (Atopic Dermatitis)	[34-36]
LGD-5552	+	+	NT	[37, 38]
Compound A (Phenyl aziridine precursor)	+	+	NT	[39, 44]
Dissociative Steroidal Compounds				
RU-Compounds	+	+	NT	[45, 46]
Lazaroids	+	+	NT	[50-53]

[1]Indicates that these compounds are tested using *in vitro* studies
[2]Indicates that these compounds are tested using *in vivo* animal models
[3]Indicates if these compounds entered human clinical trial stage
NT: Not tested.

Lazaroids

Lazaroids are 21-aminosteroids created by Pharmacia and UpJohn whose dissociative properties are accomplished through removal of the C-11 hydroxy group and replaced with a delta-9,11 bond. These 21-aminosteroids were originally developed in 1985 for their ability to inhibit of lipid peroxidation. Nearly 400 analogues have been synthesized deriving from various steroidal scaffolds with their steroid-activity-relationship being well-characterized [50]. These compounds lack GC side effects including thymus weight, body weight, hyperglycemia, and adreno-corticotropin suppression even at high doses [51, 52]. Most of the chemical and research development of these compounds has focused on maximizing their antioxidant properties for treatment in ischemia, traumatic brain injury, spinal cord injury and other indications where increased lipid peroxidation occurs. Lazaroids, unlike classic steroids are highly lipophilic compounds that intercalate into cellular membranes exerting their antioxidant effects through free radical scavenging and physiochemical interaction with cellular membranes inducing membrane stabilization [53].

There is evidence of enhanced lipid peroxidation and oxidative stress in both human and mouse dystrophin-deficient muscle that is believed to contribute to the pathogenic progression of DMD [54-57]. A recent report has shown that inhibition of lipid peroxidation with IRFI-042, a potent antioxidant, inhibited NF-κB activity and TNF-α expression in *mdx* mice along with improved strength measurements and decreased creatine kinase, and muscle necrosis [58]. These data support multiple reports of a variety of antioxidants, including lazaroids, to suppress the immune response by inhibiting NF-κB activity [59-65]. Inhibition of NF-κB resulted in reduced TNF-α, IL-6 and iNOS levels suggesting an overall suppression of the immune response.

One of the upstream events leading to increased lipid peroxidation in dystrophin-deficient skeletal muscle is the increase of cytosolic calcium due to increased membrane permeability [66]. Classic GCs reduce intracellular calcium influx in C_2C_{12} muscle cells [67]. Similarly, lazaroids also inhibit calcium influx in muscle cells but with less potency than prednisolone. Both lazaroids and prednisolone have been shown to enhance myogenesis of dystrophin deficient mouse skeletal muscle cells as determined by increase in the number of myotubes, acetylcholine receptors and α-actinin without affecting myotube size [59]. It can be hypothesized that dystrophin-deficient muscle treated with lazaroids would decrease cytosolic calcium and reduce lipid peroxidation and that these events may facilitate myogenesis. These compounds were optimized for their lipid peroxidation inhibitory effects for post-ischemic/post-traumatic injuries. However, it stands to reason that the structure-activity-relationship could be altered to better the NF-κB inhibition potency and to improve its anti-inflammatory properties thus tailoring it to inflammatory muscle diseases. The list of potential alternative compounds tested in various *in vitro* and *in vivo* studies are given in Table **3**.

SUMMARY

The therapeutic effects of GCs in the treatment of inflammatory muscle diseases have been known since the 1950's well before their mechanisms of action had begun to be understood. Prednisone and similar analogues have become the standard-of-care for genetic and autoimmune muscle diseases. However, more targeted anti-inflammatory drugs fail to prove as efficacious suggesting that it is not merely the anti-inflammatory properties of GCs that are responsible for their therapeutic effects. With a better understanding of GC's pleiotropic biological response effects, GR modulators are being designed to dissociate the therapeutic effects while reducing adverse effects. Most of the development for a safer steroid has been focused on dissociative nonsteroidal compounds, but the dissociative steroidal lazaroids also show promise as a potential new therapy for indications where GCs are standard-of-care. However, it has not yet been determined how the

improved *in vivo* therapeutic/side effect ratios in animals will translate into humans and what effect long term interference with the transactivation properties of GCs have on the safety profile.

ACKNOWLEDGEMENTS

Dr. Nagaraju is supported by the National Institutes of Health grants (R01-AR050478 and 5U54HD053177), and the Department of Defense (W81XWH-05-1-0616). ReveraGen BioPharma is supported by the National Institutes of Health, the Foundation to Eradicate Duchenne, the Muscular Dystrophy Association Venture philanthropy.

Dr. Nagaraju has stock ownership in ReveraGen BioPharma which is formerly known as Validus Biopharma.

REFERENCES

[1] Fisher, I.; Abraham, D.; Bouri, K.; Hoffman, E. P.; Muntoni, F. and Morgan, J. (2005) Prednisolone-induced changes in dystrophic skeletal muscle. *FASEB J.,* **19**, 834-836.

[2] Petrof, B. J.; Shrager, J. B.; Stedman, H. H.; Kelly, A. M. and Sweeney, H. L. (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. USA,* **90**, 3710-3714.

[3] Kumar, A. and Boriek, A. M. (2003) Mechanical stress activates the nuclear factor-kappaB pathway in skeletal muscle fibers: a possible role in Duchenne muscular dystrophy. *FASEB J.,* **17**, 386-396.

[4] Spencer, M. J.; Montecino-Rodriguez, E.; Dorshkind, K. and Tidball, J. G. (2001) Helper (CD4(+)) and cytotoxic (CD8(+)) T cells promote the pathology of dystrophin-deficient muscle. *Clin. Immunol.,* **98**, 235-243.

[5] Chen, Y. W.; Nagaraju, K.; Bakay, M.; McIntyre, O.; Rawat, R.; Shi, R. and Hoffman, E. P. (2005) Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. *Neurology,* **65**, 826-834.

[6] McDouall, R. M.; Dunn, M. J. and Dubowitz, V. (1990) Nature of the mononuclear infiltrate and the mechanism of muscle damage in juvenile dermatomyositis and Duchenne muscular dystrophy. *J. Neurol. Sci.,* **99**, 199-217.

[7] Evans, N. P.; Misyak, S. A.; Robertson, J. L.; Bassaganya-Riera, J. and Grange, R. W. (2009) Immune-mediated mechanisms potentially regulate the disease time-course of duchenne muscular dystrophy and provide targets for therapeutic intervention. *PM R.,* **1**, 755-768.

[8] Hodgetts, S.; Radley, H.; Davies, M. and Grounds, M. D. (2006) Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. *Neuromuscul. Disord.,* **16**, 591-602.

[9] Gorospe, J. R.; Tharp, M. D.; Hinckley, J.; Kornegay, J. N. and Hoffman, E. P. (1994) A role for mast cells in the progression of Duchenne muscular dystrophy? Correlations in dystrophin-deficient humans, dogs, and mice. *J. Neurol. Sci.,* **122**, 44-56.

[10] Appleyard, S. T.; Dunn, M. J.; Dubowitz, V. and Rose, M. L. (1985) Increased expression of HLA ABC class I antigens by muscle fibres in Duchenne muscular dystrophy, inflammatory myopathy, and other neuromuscular disorders. *Lancet,* **1**, 361-363.

[11] McDouall, R. M.; Dunn, M. J. and Dubowitz, V. (1989) Expression of class I and class II MHC antigens in neuromuscular diseases, *J. Neurol. Sci.,* **89**, 213-226.

[12] Nagaraju, K. and Lundberg, I. (2009) Inflammatory diseases of muscle and other myopathies in *Kelly's Text book of Rheumatology* (Firestein, G. S., Budd, R. C., Harris, J., E.D., McInnes, I. B., Ruddy, S. & Sergent, J. S., eds) pp. 1353-1380, Saunders, Elsevier, Philadelphia.

[13] Nagaraju, K.; Raben, N.; Loeffler, L.; Parker, T.; Rochon, P. J.; Lee, E.; Danning, C.; Wada, R.; Thompson, C.; Bahtiyar, G.; Craft, J.; Hooft Van Huijsduijnen, R. and Plotz, P. (2000) Conditional up-regulation of MHC class I in skeletal muscle leads to self-sustaining autoimmune myositis and myositis-specific autoantibodies. *Proc. Natl. Acad. Sci. USA,* **97**, 9209-9214.

[14] Nagaraju, K.; Casciola-Rosen, L.; Lundberg, I.; Rawat, R.; Cutting, S.; Thapliyal, R.; Chang, J.; Dwivedi, S.; Mitsak, M.; Chen, Y. W.; Plotz, P.; Rosen, A.; Hoffman, E. and Raben, N. (2005) Activation of the endoplasmic reticulum stress response in autoimmune myositis: potential role in muscle fiber damage and dysfunction. *Arthritis Rheum.,* **52**, 1824-1835.

[15] Henriques-Pons, A. and Nagaraju, K. (2009) Nonimmune mechanisms of muscle damage in myositis: role of the endoplasmic reticulum stress response and autophagy in the disease pathogenesis. *Curr. Opin. Rheumatol.,* **21**, 581-587.

[16] Beenakker, E. A.; Fock, J. M.; Van Tol, M. J.; Maurits, N. M.; Koopman, H. M.; Brouwer, O. F. and Van der Hoeven, J. H. (2005) Intermittent prednisone therapy in Duchenne muscular dystrophy: a randomized controlled trial, *Arch. Neurol.,* **62**, 128-132.

[17] Distad, B. J.; Amato, A. A. and Weiss, M. D. Inflammatory myopathies. *Curr. Treat. Options Neurol.,* **13**, 119-130.

[18] Lowenberg, M.; Stahn, C.; Hommes, D. W. and Buttgereit, F. (2008) Novel insights into mechanisms of glucocorticoid action and the development of new glucocorticoid receptor ligands. *Steroids,* **73**, 1025-1029.

[19] Buttgereit, F. and Scheffold, A. (2002) Rapid glucocorticoid effects on immune cells. *Steroids,* **67**, 529-534.

[20] Buttgereit, F. (2000) Mechanisms and clinical relevance of nongenomic glucocorticoid actions. *Z. Rheumatol.,* **59(**Suppl 2), II/ 119-123.

[21] Croxtall, J. D.; Choudhury, Q. and Flower, R. J. (2000) Glucocorticoids act within minutes to inhibit recruitment of signalling factors to activated EGF receptors through a receptor-dependent, transcription-independent mechanism. *Br. J. Pharmacol.,* **130**, 289-298.

[22] Mendell, J. R.; Moxley, R. T.; Griggs, R. C.; Brooke, M. H.; Fenichel, G. M.; Miller, J. P.; King, W.; Signore, L.; Pandya, S.; Florence, J.; *et al.* (1989) Randomized, double-blind six-month trial of prednisone in Duchenne's muscular dystrophy. *N. Engl. J. Med.,* **320**, 1592-1597.

[23] Manzur, A. Y.; Kuntzer, T.; Pike, M. and Swan, A. (2008) Glucocorticoid corticosteroids for Duchenne muscular dystrophy. *Cochrane Database Syst. Rev.*, CD003725.

[24] Baltgalvis, K. A.; Call, J. A.; Nikas, J. B. and Lowe, D. A. (2009) Effects of prednisolone on skeletal muscle contractility in mdx mice. *Muscle Nerve.,* **40**, 443-454.

[25] Biedasek, K.; Andres, J.; Mai, K.; Adams, S.; Spuler, S.; Fielitz, J. and Spranger, J. (2011) Skeletal muscle 11beta-HSD1 controls glucocorticoid-induced proteolysis and expression of E3 ubiquitin ligases atrogin-1 and MuRF-1, *PLoS One,* **6**, e16674.

[26] Schakman, O.; Gilson, H.; Kalista, S. and Thissen, J. P. (2009) Mechanisms of muscle atrophy induced by glucocorticoids. *Horm. Res., 72* Suppl 1, 36-41.

[27] Shimizu, N.; Yoshikawa, N.; Ito, N.; Maruyama, T.; Suzuki, Y.; Takeda, S.; Nakae, J.; Tagata, Y.; Nishitani, S.; Takehana, K.; Sano, M.; Fukuda, K.; Suematsu, M.; Morimoto, C. and Tanaka, H. Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. *Cell Metab.,* **13**, 170-182.

[28] Guerron, A. D.; Rawat, R.; Sali, A.; Spurney, C. F.; Pistilli, E.; Cha, H. J.; Pandey, G. S.; Gernapudi, R.; Francia, D.; Farajian, V.; Escolar, D. M.; Bossi, L.; Becker, M.; Zerr, P.; de la Porte, S.; Gordish-Dressman, H.; Partridge, T.; Hoffman, E. P. and Nagaraju, K. (2011) Functional and molecular effects of arginine butyrate and prednisone on muscle and heart in the mdx mouse model of Duchenne Muscular Dystrophy. *PLoS One,* **5**, e11220.

[29] Schacke, H.; Docke, W. D. and Asadullah, K. (2002) Mechanisms involved in the side effects of glucocorticoids, *Pharmacol. Ther.,* **96**, 23-43.

[30] Cordeiro, A. C. and Isenberg, D. A. (2006) Treatment of inflammatory myopathies, *Postgrad. Med. J.,* **82**, 417-424.

[31] Coghlan, M. J.; Jacobson, P. B.; Lane, B.; Nakane, M.; Lin, C. W.; Elmore, S. W.; Kym, P. R.; Luly, J. R.; Carter, G. W.; Turner, R.; Tyree, C. M.; Hu, J.; Elgort, M.; Rosen, J. and Miner, J. N. (2003) A novel antiinflammatory maintains glucocorticoid efficacy with reduced side effects, *Mol. Endocrinol.,* **17**, 860-869.

[32] Elmore, S. W.; Coghlan, M. J.; Anderson, D. D.; Pratt, J. K.; Green, B. E.; Wang, A. X.; Stashko, M. A.; Lin, C. W.; Tyree, C. M.; Miner, J. N.; Jacobson, P. B.; Wilcox, D. M. and Lane, B. C. (2001) Nonsteroidal selective glucocorticoid modulators: the effect of C-5 alkyl substitution on the transcriptional activation/repression

profile of 2,5-dihydro-10-methoxy-2,2,4-trimethyl-1H-[1]benzopyrano [3,4-f]quinolines. *J. Med. Chem.,* **44**, 4481-4491.

[33] Owen, H. C.; Miner, J. N.; Ahmed, S. F. Farquharson, C. (2007) The growth plate sparing effects of the selective glucocorticoid receptor modulator, AL-438, *Mol. Cell Endocrinol.,* **264**, 164-170.

[34] Schacke, H.; Schottelius, A.; Docke, W. D.; Strehlke, P.; Jaroch, S.; Schmees, N.; Rehwinkel, H.; Hennekes, H. and Asadullah, K. (2004) Dissociation of transactivation from transrepression by a selective glucocorticoid receptor agonist leads to separation of therapeutic effects from side effects. *Proc. Natl. Acad. Sci. USA,* **101**, 227-232.

[35] Humphrey, E. L.; Williams, J. H.; Davie, M. W. and Marshall, M. J. (2006) Effects of dissociated glucocorticoids on OPG and RANKL in osteoblastic cells. *Bone, **38***, 652-661.

[36] Schacke, H.; Zollner, T. M.; Docke, W. D.; Rehwinkel, H.; Jaroch, S.; Skuballa, W.; Neuhaus, R.; May, E.; Zugel, U. and Asadullah, K. (2009) Characterization of ZK 245186, a novel, selective glucocorticoid receptor agonist for the topical treatment of inflammatory skin diseases. *Br. J. Pharmacol.,* **158**, 1088-1103.

[37] Miner, J. N.; Ardecky, B.; Benbatoul, K.; Griffiths, K.; Larson, C. J.; Mais, D. E.; Marschke, K.; Rosen, J.; Vajda, E.; Zhi, L. and Negro-Vilar, A. (2007) Antiinflammatory glucocorticoid receptor ligand with reduced side effects exhibits an altered protein-protein interaction profile. *Proc. Natl. Acad. Sci. USA,* **104**, 19244-19249.

[38] Lopez, F. J.; Ardecky, R. J.; Bebo, B.; Benbatoul, K.; De Grandpre, L.; Liu, S.; Leibowitz, M. D.; Marschke, K.; Rosen, J.; Rungta, D.; Viveros, H. O.; Yen, W. C.; Zhi, L.; Negro-Vilar, A. and Miner, J. N. (2008) LGD-5552, an antiinflammatory glucocorticoid receptor ligand with reduced side effects, *in vivo*. *Endocrinology,* **149**, 2080-2089.

[39] De Bosscher, K.; Vanden Berghe, W.; Beck, I. M.; Van Molle, W.; Hennuyer, N.; Hapgood, J.; Libert, C.; Staels, B.; Louw, A. and Haegeman, G. (2005) A fully dissociated compound of plant origin for inflammatory gene repression. *Proc. Natl. Acad. Sci. USA,* **102**, 15827-15832.

[40] Dewint, P.; Gossye, V.; De Bosscher, K.; Vanden Berghe, W.; Van Beneden, K.; Deforce, D.; Van Calenbergh, S.; Muller-Ladner, U.; Vander Cruyssen, B.; Verbruggen, G.; Haegeman, G. and Elewaut, D. (2008) A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis. *J. Immunol.,* **180**, 2608-2615.

[41] Wust, S.; Tischner, D.; John, M.; Tuckermann, J. P.; Menzfeld, C.; Hanisch, U. K.; van den Brandt, J.; Luhder, F. and Reichardt, H. M. (2009) Therapeutic and adverse effects of a non-steroidal glucocorticoid receptor ligand in a mouse model of multiple sclerosis. *PLoS One.,* **4**, e8202.

[42] Robertson, S.; Allie-Reid, F.; Vanden Berghe, W.; Visser, K.; Binder, A.; Africander, D.; Vismer, M.; De Bosscher, K.; Hapgood, J.; Haegeman, G. and Louw, A. Abrogation of glucocorticoid receptor dimerization correlates with dissociated glucocorticoid behavior of compound a. *J. Biol. Chem.,* **285**, 8061-8075.

[43] Zhang, Z.; Zhang, Z. Y. and Schluesener, H. J. (2009) Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects. *J. Immunol.,* **183**, 3081-3091.

[44] van Loo, G.; Sze, M.; Bougarne, N.; Praet, J.; Mc Guire, C.; Ullrich, A.; Haegeman, G.; Prinz, M.; Beyaert, R. and De Bosscher, K. Antiinflammatory properties of a plant-derived nonsteroidal, dissociated glucocorticoid receptor modulator in experimental autoimmune encephalomyelitis. *Mol. Endocrinol.,* **24**, 310-322.

[45] Vayssiere, B. M.; Dupont, S.; Choquart, A.; Petit, F.; Garcia, T.; Marchandeau, C.; Gronemeyer, H. and Resche-Rigon, M. (1997) Synthetic glucocorticoids that dissociate transactivation and AP-1 transrepression exhibit antiinflammatory activity *in vivo*, *Mol. Endocrinol.,* **11**, 1245-1255.

[46] Belvisi, M. G.; Wicks, S. L.; Battram, C. H.; Bottoms, S. E.; Redford, J. E.; Woodman, P.; Brown, T. J.; Webber, S. E. and Foster, M. L. (2001) Therapeutic benefit of a dissociated glucocorticoid and the relevance of *in vitro* separation of transrepression from transactivation activity. *J. Immunol.,* **166**, 1975-1982.

[47] Schacke, H.; Berger, M.; Rehwinkel, H. and Asadullah, K. (2007) Selective glucocorticoid receptor agonists (SEGRAs): novel ligands with an improved therapeutic index. *Mol. Cell Endocrinol.,* **275**, 109-117.

[48] Chen, T. (2008) Nuclear receptor drug discovery. *Curr. Opin. Chem. Biol.,* **12**, 418-426.

[49] De Bosscher, K.; Beck, I. M. and Haegeman, G. (2010) Classic glucocorticoids versus non-steroidal glucocorticoid receptor modulators: survival of the fittest regulator of the immune system?, *Brain Behav. Immun.,* **24**, 1035-1042.

[50] Hall, E. D.; Braughler, J. M. and McCall, J. M. (1990) Role of oxygen radicals in stroke: effects of the 21-aminosteroids (lazaroids). A novel class of antioxidants. *Prog. Clin. Biol. Res.,* **361**, 351-362.

[51] McCall, J. M.; Braughler, J. M. and Hall, E. D. (1987) A new class of compounds for stroke and trauma: effects of 21-aminosteroids on lipid peroxidation. *Acta Anaesthesiol. Belg.,* **38**, 417-420.

[52] Braughler, J. M.; Chase, R. L.; Neff, G. L.; Yonkers, P. A.; Day, J. S.; Hall, E. D.; Sethy, V. H. and Lahti, R. A. (1988) A new 21-aminosteroid antioxidant lacking glucocorticoid activity stimulates adrenocorticotropin secretion and blocks arachidonic acid release from mouse pituitary tumor (AtT-20) cells. *J. Pharmacol. Exp. Ther.,* **244**, 423-427.

[53] Hall, E. D.; McCall, J. M. and Means, E. D. (1994) Therapeutic potential of the lazaroids (21-aminosteroids) in acute central nervous system trauma, ischemia and subarachnoid hemorrhage. *Adv. Pharmacol.,* **28**, 221-268.

[54] Spassov, A.; Gredes, T.; Gedrange, T.; Pavlovic, D.; Lupp, A. and Kunert-Keil, C. (2011) Increased oxidative stress in dystrophin deficient (mdx) mice masticatory muscles. *Exp. Toxicol. Pathol*., **63**, 549-552.

[55] Tidball, J. G. and Wehling-Henricks, M. (2007) The role of free radicals in the pathophysiology of muscular dystrophy. *J. Appl. Physiol.,* **102**, 1677-1686.

[56] Ragusa, R. J.; Chow, C. K. and Porter, J. D. (1997) Oxidative stress as a potential pathogenic mechanism in an animal model of Duchenne muscular dystrophy. *Neuromuscul. Disord.,* **7**, 379-386.

[57] Rodriguez, M. C. and Tarnopolsky, M. A. (2003) Patients with dystrophinopathy show evidence of increased oxidative stress. *Free Radic Biol. Med.,* **34**, 1217-1220.

[58] Messina, S.; Bitto, A.; Aguennouz, M.; Minutoli, L.; Monici, M. C.; Altavilla, D.; Squadrito, F. and Vita, G. (2006) Nuclear factor kappa-B blockade reduces skeletal muscle degeneration and enhances muscle function in Mdx mice. *Exp. Neurol.,* **198**, 234-241.

[59] Metzinger, L.; Passaquin, A. C.; Vernier, A.; Thiriet, N.; Warter, J. M. and Poindron, P. (1994) Lazaroids enhance skeletal myogenesis in primary cultures of dystrophin-deficient mdx mice. *J. Neurol. Sci.,* **126**, 138-145.

[60] Fukuma, K.; Marubayashi, S.; Okada, K.; Yamada, K.; Kimura, A. and Dohi, K. (1999) Effect of lazaroid U-74389G and methylprednisolone on endotoxin-induced shock in mice. *Surgery,* **125**, 421-430.

[61] Marubayashi, S.; Okada, K.; Fukuma, K.; Tashiro, H.; Kitayama, T.; Hayamizu, K.; Dohi, K. and Asahara, T. (2002) Effect of lazaroids on NF-kappa B activation of Kupffer cells in liver preservation, *Transplant. Proc.,* **34**, 2662-2663.

[62] Okada, K.; Marubayashi, S.; Fukuma, K.; Yamada, K. and Dohi, K. (2000) Effect of the 21-aminosteroid on nuclear factor-kappa B activation of Kupffer cells in endotoxin shock. *Surgery,* **127**, 79-86.

[63] Hsieh, H. M.; Wu, W. M. and Hu, M. L. Genistein attenuates D-galactose-induced oxidative damage through decreased reactive oxygen species and NF-kappaB binding activity in neuronal PC12 cells. *Life Sci.,* **88**, 82-88.

[64] Tunon, M. J.; Garcia-Mediavilla, M. V.; Sanchez-Campos, S. and Gonzalez-Gallego, J. (2009) Potential of flavonoids as anti-inflammatory agents: modulation of pro-inflammatory gene expression and signal transduction pathways. *Curr. Drug Metab.,* **10**, 256-271.

[65] Pinkus, R.; Weiner, L. M. and Daniel, V. (1996) Role of oxidants and antioxidants in the induction of AP-1, NF-kappaB, and glutathione S-transferase gene expression. *J. Biol. Chem.,* **271**, 13422-13429.

[66] Allen, D. G. and Whitehead, N. P. Duchenne muscular dystrophy--what causes the increased membrane permeability in skeletal muscle?. *Int. J. Biochem. Cell Biol.,* **43**, 290-294.

[67] Passaquin, A. C.; Lhote, P. and Ruegg, U. T. (1998) Calcium influx inhibition by steroids and analogs in C2C12 skeletal muscle cells. *Br. J. Pharmacol.,* **124**, 1751-1759.

[68] Weinblatt, M. E. (1995) Efficacy of methotrexate in rheumatoid arthritis. *Br. J. Rheumatol., **34** Suppl 2*, 43-48.

[69] Callen, J. P. (2000) Dermatomyositis. *Lancet,* **355**, 53-57.

[70] Mastaglia, F. L. and Zilko, P. J. (2003) Inflammatory myopathies: how to treat the difficult cases. *J. Clin. Neurosci.,* **10**, 99-101.

[71] Griggs, R. C.; Moxley, R. T. 3rd; Mendell, J. R.; Fenichel, G. M.; Brooke, M. H.; Pestronk, A.; Miller, J. P.; Cwik, V. A., Pandya, S., Robison, J. & *et al*. (1993) Duchenne dystrophy: randomized, controlled trial of prednisone (18 months) and azathioprine (12 months). *Neurology,* **43**, 520-527.

[72] Kirschner, J.; Schessl, J.; Schara, U.; Reitter, B.; Stettner, G. M.; Hobbiebrunken, E.; Wilichowski, E.; Bernert, G.; Weiss, S.; Stehling, F.; Wiegand, G.; Muller-Felber, W.; Thiele, S.; Grieben, U.; von der Hagen, M.; Lutschg, J.; Schmoor, C.; Ihorst, G. and Korinthenberg, R. (2010) Treatment of Duchenne muscular dystrophy with ciclosporin A: a randomised, double-blind, placebo-controlled multicentre trial *Lancet Neurol., **9***, 1053-1059.

[73] Dalakas, M. C.; Illa, I.; Dambrosia, J. M.; Soueidan, S. A.; Stein, D. P.; Otero, C.; Dinsmore, S. T. and McCrosky, S. (1993) A controlled trial of high-dose intravenous immune globulin infusions as treatment for dermatomyositis. *N. Engl. J. Med.,* **329**, 1993-2000.

[74] Hengstman, G. J.; van den Hoogen, F. H.; Barrera, P.; Netea, M. G.; Pieterse, A.; van de Putte, L. B. and van Engelen, B. G. (2003) Successful treatment of dermatomyositis and polymyositis with anti-tumor-necrosis-factor-alpha: preliminary observations. *Eur. Neurol.,* **50**, 10-15.

[75] Majmudar, S.; Hall, H. A. and Zimmermann, B. (2009) Treatment of adult inflammatory myositis with rituximab: an emerging therapy for refractory patients. *J. Clin. Rheumatol.,* **15**, 338-340.

Received: 25 May, **2011** **Accepted: 19 October**, **2011**